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IMPROVED METHOD FOR THE PREPARATION OF CRYSTALLINE OXYHEMOGLOBIN FREE FROM CATALASE AND METHEMOGLOBIN

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(Received for publication, January 27, 1954)

There are significant differences in solubilities among oxyhemoglobins of different species. It is well known that bovine oxyhemoglobin, as those of man and sheep, have higher solubilities than those of horse and rat. On account of its higher solubility, the crystalline bovine oxyhemoglobin preparation has hitherto been only rarely obtained.

On horse oxyhemoglobin, however, a number of reports was published as by Hoppe-Seyler (*1*), Heidelberger (*2*), Ferry and Green (*3*) and Haurowitz (*4*) *etc.* Though much attention was payed in their reports on the methemoglobin (hemiglobin) contents of their preparations, the catalase content was mostly overlooked.

The authors were able to obtain, by relatively simple methods, pure preparations of bovine and horse oxyhemoglobin crystals which were free from catalase.

EXPERIMENTAL

I. Procedures for the Crystalline Preparation

Bovine Oxyhemoglobin—Freshly separated bovine erythrocytes, repeatedly (5–6 times) washed with physiological saline solution, were hemolyzed by addition of an equal volume of distilled water and destromatized by vigorous shaking with a equal volume of ether.

By centrifuging, the mixture separated in three layers, the stroma being concentrated in the middle layer. The bottom layer of the hemolyzate solution was taken out by syphoning.

Cold ethanol was then carefully dropped into the hemolyzate solution which was kept stirred in an ice bath until the ethanol concentration reached 30 per cent. On keeping the mixture in an ice box for 24 hours, fine needles were obtained (Plate 1).

The crystalls were washed twice 30 per cent ethanol saturated with ether. The crystals were then redissolved in a minimum volume of water saturated with

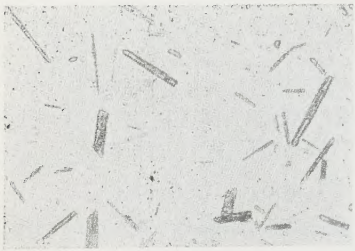

PLATE 1. Crystalline bovine HbO_2.

ether and recrystallized by the addition of ethanol, the concentration of the latter being reduced to 15–20 per cent by repeating the recrystallization. After 3 times of recrystallization, the preparation were completely free from catalase. The reduction of catalase content during repeated recrystallization is shown in Fig. 1.

Catalase content was determined by Euler and Joesephson's (*5*) method. The final yield of the crystalline preparation was about 25 per cent.

Horse Oxyhemoglobin—

(i) Destromatized horse hemolyzate obtained by the same procedure as above was dialysed against cold distilled water for 24 hours. Triclinic plates (Plate 2) were then obtained, which were free from catalase. The method is simple, but its reproducibility was unsatisfactory and often with poorer yields.

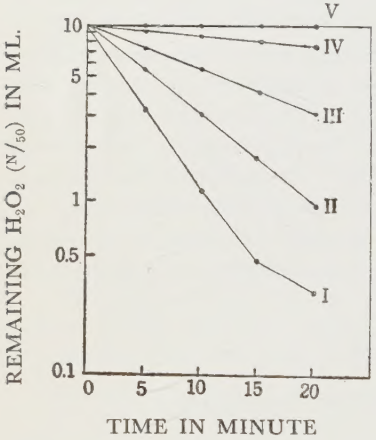

FIG. 1. Determination of catalase by Euler and Josephson's method. I. Hemolyzate II. Solution of crystalline HbO_2 III. HbO_2 after first recrystallization IV. HbO_2 after second recrystallization V. HbO_2 after third recrystallization. Each HbO_2 concentration: 5×10^{-5} *M*.

(ii) Destromatized horse hemolyzate was gently bubbled with a gas mixture of oxygen and corbon dioxide (1 : 4) which was introduced through a glass capillary at 0°C according to Heidelberger (*2*), until the pH of the hemolyzate was reduced to about 6.4. Total gas volume aerated amounted up to 4 times of the volume of hemolyzate. The capillary must be made as fine as possible.

By keeping the solution for 48 hours in an ice box, triclinic plates were obtained. The relationship between the aerated gas volume, pH of the solution and yields of crystal is shown in Fig. 2. Maximum yield was about 70 per cent. The yield were calculated from the spectrophotometric determination of remaining oxyhemoglobin in the supernatant.

The crystals which were washed several times with cold distilled water were found to be quite free from catalase.

The crystals obtained in (i) and (ii) were redissolved in a small portion of *M*/10 Na_2CO_3 or *M*/10 Na_2HPO_4 and recrystallized by dialysis or by aeration with the

above mentioned gas mixture.

II. Analysis of the Preparation

The crystalline bovine and horse oxyhemoglobins obtained as above were dissolved in distilled water and their optical absorptions were measured. By the addition of sodium fluoride to the oxyhemoglobin solution, no absorption change was observed. Our preparation was found, thus, to be practically free from methemoglobin. Catalase also could not be detected in our preparation as already mentioned.

A dry preparation of horse oxyhemoglobin was obtained by freezing in vacum and by further evacuation at 6mm. -Hg., at 110°C in Abderhalden's desiccator for 4 hours until its weight became constant. Decrease in weight was about 10 per cent of the liophylized preparation. The result was well in accordance with that reported by Haurowitz (*4*). Nitrogen and iron contents of our preparation were determined by the micro Kjeldahl method and by Yamada's *o*-phenanthrolin method (*6*), respectively.

N: 16.5 per cent Fe: 0.341 per cent Molecular weight of horse hemoglobin could thus be calculated to be 65,500.

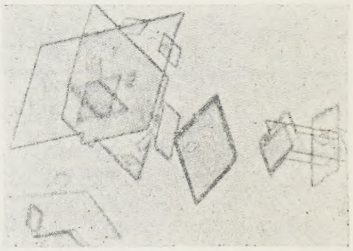

PLATE 2. Crystalline horse HbO_2.

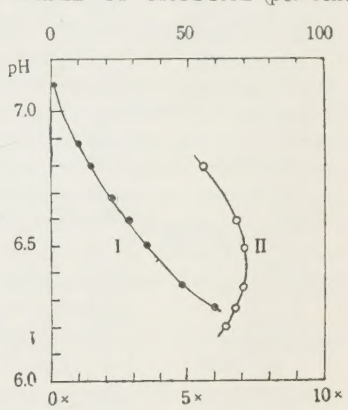

FIG. 2. Relationship between the aerated gas volume, pH of the solution and the yields of crystal.
I. pH vs. gas volume —●—
II. pH vs. yields —○—

III. Absorption Maxima and Milli-molar-extinction of Our Preparation and Its Derivatives

Alkali-denatured globin hemochrome solution was prepared as follows: crystalline horse oxyhemoglobin was dissolved in sodium hydroxide solution (final concentration 1 per cent), a small amount of sodium dithionite was added to the solution and the mixture was covered with liquid paraffin. Absorption curve was recorded after 15 minutes. Two distinguished absorption bands were observed at 559 and 528 mμ. From the observed optical density and the Fe content, it was found that $\varepsilon_{\text{mM}}^{559}=30.6$. This value was used as the standard for the calculation of molar extinctions of other derivatives. The absorption maxima and the milli-molar extinctions

TABLE I

Optical Constants of Hemoglobin Derivatives

Derivatives	Figures determined by the present authors		Data in the literature (7)		
	$m\mu$	εmM	$m\mu$	εmM	
HbO_2	542	14.3	540–542	14.2–15.3	Drabkin (1946)
(dist.	578	15.0	576–578	15.1–16.2	,, (1935–6)
water)	414	132	412–415	125 –128.5	Hicks (1929)
Hemochrome	559	30.6	555–560	25 –30.9	Drabkin (1942)
alkali-denatured	528	14.5	528–530	10.7–14.8	,, ,,
globin (NaOH :1%)	425	156	424	110	Holden (1932)
reduced Hb	556	13.5	555	12.9–13.6	Drabkin (1935–6)
($N/10$ Na_2CO_3)	430	144	430	118 –134	,, ,,
HbCO	540	14.7	538–540	13.7–15.0	Drabkin (1942)
($N/10$	570	14.4	568–572	14.1–15.3	Heilmayer (1943)
Na_2CO_3)	420	197	418	154	Hicks and Holden (1929)
HiCN	540	11.4	540	10.8–11.5	Kennedy (1927)
(KCN:	421	117	412–41	692 –104	Drabkin (1935–6)
1.7×10^{-2} M)					Howell (1921)
HiF	607	9.5	—	—	
(NaF : 0.5 M)	403	143	—	—	
HiOH	577	8.4	577	8.5	Horecker (1943)
(Na_2CO_3:	540	9.4	540	9.7	,, ,,
pH 10.4)	412	83.0	411	71 – 90	Hicks (1929)
Hi^+	630	3.8	630	3.7 –3.8	Horecker (1943)
($2M/45$ KH_2PO_4:	500	9.4	500	9.5	Kennedy (1927)
pH 5.91)	406	168	405–407	134 –154	Hicks and Holden (1929)

isosbestic points between HiOH and Hi^+, Hi^+.....methemoglobin in acidic side — 618 and 521 mμ

thus determined of various hemoglobin derivatives, i.e., oxyhemoglobin, alkali-denatured globin hemochrome, reduced hemoglobin, carbon mono-oxide hemoglobin, cyan methemoglobin, fluoride methemoglobin and methemoglobins (both in alkaline and acidic sides), are recorded in TABLE I. Determinations were carried out by the "Hitachi" Photoelectric Spectro-photometer (for vissible range).

IV. Rates of Oxyhemoglobin Autoxidation Induced by Sodium Benzoate in the Solutions of Crystalline Preparation and of Hemolyzate

Anson and Mirsky (9) reported that the absorption curve of methemoglobin

changed reversibly by the addition of sodium salicylate. A similar effect was discovered by Holden (*10*) with sodium benzoate and patassium iodide and he named the phenomenon "perturbation" making a distinction from "denaturation." Recently K. Tsushima (*11*) in our laboratory reported that the rate of oxyhemoglobin autoxidation was accelerated by sodium benzoate through a "perturbating" effect of the latter upon protein part of hemoglobin. If the protein part of our crystalline oxyhemoglobin might have been denatured, even slightly, in the course of the preparative procedure, the rate of autoxidation induced by sodium benzoate would be higher as compared with that of the native oxyhemoglobin (hemolyzate). To obtain some information regarding this point, the following experiments were carried out.

Destromatized bovine and horse hemolyzates and solutions of both crystalline preparations were incubated at 30°C. After 10 minutes, sodium benzoate solution, which had been incubated similarly, were added to these oxyhemoglobin solutions. The final concentration of oxyhemoglobin was $5.0\times10^{-5}M$ and that of sodium benzoate was $1.2\,M$. Absorption curves of the reaction mixtures turned with the process of autoxidation to those of a mixture consisting of parahaematin (hemichrome) and oxyhemoglobin. When KCN was added to this reaction mixture, the absorption figures turned to those of a mixture of cyan-methemoglobin ann oxyhemoglobin. An aliquot portion of the reaction mixture was taken out every 5 minutes and KCN was added to them. The optical densities at 578 mμ (absorption maximum of oxyhemoglobin) was then determined. Percentages of the autoxidized portion of hemoglobin were calculated by the use of the nomogram established with a series of varied mixtures of cyan-methemoglobin and oxyhemoglobin.

The reaction was a first order reaction in respect to the oxyhemoglobin concentration.

The results are shown in Figs. 3 and 4. It was proved that our crystalline horse oxyhemoglobin remains fairly in native state, while that of bovine seems to have been affected to some extent in its protein part by the repetition of recrystallization.

V. Autoxidation of Oxyhemoglobin Solutions Standing in Refrigerator

Autoxidation in the stock solutions of the crystals and the destromatized hemolyzates were compared by keeping them in a refrigerator. The determinations were carried out in the same manner as described in *Section IV*. 100 units of penicillin was added per ml. of the solution for protection from bacterial contamination. The results are given in Fig. 5.

DISCUSSION

Although the crystalline bovine oxyhemoglobin may be obtained by salting out method, the procedure of removing the salts from the preparation is rather tedious and onerous. In the ethanol-ether method reported here, crystallization is simple and the elimination of the salts

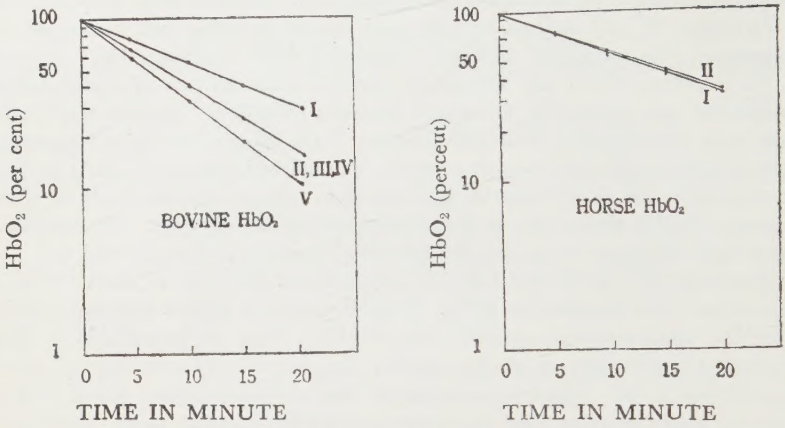

Fig. 3. Autoxidation of bovine HbO_2 induced by Na benzoate Final concentration of Na benzoate: 1.2 *M*, T=30°C.

I. Destromatized bovine hemolyzate II. Solution of crystalline bovine HbO_2 (crystallized in 30% ethanol) III. HbO_2 after recrystallization (crystallized in 30% ethanol) IV. HbO_2 after second recrystallization (crystallized in 20% ethanol) V. HbO_2 after third recrystallization (crystallized in 15% ethanol) Final concentration of HbO_2: 5×10^{-5} *M*.

Fig. 4. Autoxidation of horse HbO_2 induced by Na benzoate. Final concentration of Na benzoate: 1.2 *M*, T=30°C.

I. Destromatized horse hemolyzate II. Solution of crystalline equine HbO_2 Final concentration of HbO_2: 5×10^{-5} *M*.

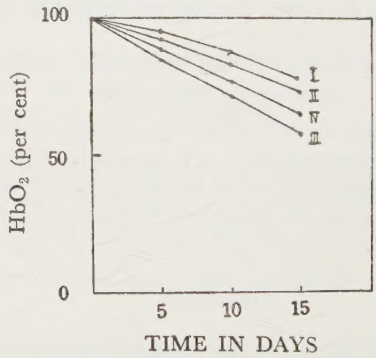

Fig. 5. Autoxidation during the storage in a refrigerator

I. Bovine hemolyzate. II. Equine hemolyzate. III. Solution of crystalline bovine HbO_2. IV. Solution of crystalline equine HbO_2. Concentration: 5×10^{-5} *M*.

is, of course, unnecessary. The only difficulty lies in the removal of catalase from the preparation. In the methods using organic solvents, proteins may be partially affected risking their denaturation as pointed out by Haurowitz (*4*). Among the methods so far reported for the crystalline preparation of horse oxyhemoglobin, Heidelberger's method with bubbling of gas mixture seems to be the most justifiable one, since both Hoppe-Seyler's ethanol method and Ferry and Green's hydrochloric acid method have some weak point in risking denaturation of the protein part. In authors' method, ether was used for destromatization instead of toluol. Ether is more easily removable than toluol. In the present method, maximum yield was obtained at pH 6.5–6.4 rather than at pH 6.65 *i.e.* the isoelectric point of oxyhemoglobin. This seems to be due to the possible evaporation of carbon dioxide during the storage in refrigerator.

SUMMARY

1. Crystalline preparations of bovine and horse oxyhemoglobin which were free from catalase and methaemoglobin, were obtained.

2. Crystalline bovine oxyhemoglobin was prepared by means of fractionation with ethanol. Crystalline horse oxyhemoglobin could be obtained merely by dialysis against cold distilled water, but, often with poorer yields and with unsatisfactory reproducibility. In its simplicity, however, Heidelberger's method was proved so far to be most convenient. The adequate conditions of the latter method were carefully studied in the present investigation.

3. The preparation of horse oxyhemoglobin prepared by Heidelberger's method remained fairly stable in its native state, while bovine oxyhemoglobin seemed to have been subjected to some denaturation in its protein part.

4. Contents of N and Fe in the horse hemoglobin prepared by us were found to be 16.5 per cent and 0.34 per cent, respectively.

5. Using the preparations obtained, the optical constants of various hemoglobin derivatives were determined.

Present investigation was undertaken as a part of the program of our laboratory: "Interrelation between the function of heme-proteins and the structural modifications of their protein parts."

We wish to thank Profs. K. Kaziro and G. Kikuchi for their suggestion and kind advices.

REFERENCES

(*1*) Hoppe-Seyler, F., Medizinisch-chemische Untersuchungen, **2**, 181 (1867)
(*2*) Heidelberger, M., *J. Biol. Chem.*, **53**, 34 (1922)
(*3*) Ferry, R.M., and Green, A.A., *J. Biol. Chem.*, **81**, 175 (1929)
(*4*) Haurowitz, F., *Z. physiol. Chem.*, **136**, 147 (1924)
(*5*) v. Euler, H., and Josephson, K., *Ann. Chem.*, **452**, 158 (1927)
(*6*) Yamada, M., *Nippon Ika. Daig. Z.*, **19**, 1093 (1952)
(*7*) Lemberg, R., and Legge, J.W., Hematin compounds and bile pigments. Interscience Publishers, N.Y. p. 228 (1949)
(*8*) Anson, M.L., and Mirsky, A.E., *J. Gen. Physiol.*, **13**, 129 (1929)
(*9*) Anson, M.L., and Mirsky, A.E., *J. Gen. Physiol.*, **17**, 399 (1934)
(*10*) Holden, H.F., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 47 (1947)
(*11*) Tsushima, K., *J. Biochem.*, **41**, 359 (1954)

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THE EFFECT OF AGE AND ADRENALECTOMY ON THE D-AMINO ACID OXIDASE ACTIVITY OF RAT LIVER

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(Received for publication, February 11, 1954)

The D-amino acid oxidase (DAO) of rat liver has been shown to be influenced by factors affecting endocrine function and by diet. Klein (*1*) has shown that the feeding of desiccated thyroid increases and thyroidectomy decreases its activity. Umbreit and Tonhazy (*2*) have observed that the liver DAO activity is decreased by adrenalectomy and that the normal level of activity is restored by the administration of cortisone. The DAO activity can also be reduced below normal by nutritional deficiencies (*3*), *i.e.* riboflavin or protein.

In the present study, the DAO activity has been found to vary with the age of the rat and that its intracellular distribution is altered by adrenalectomy.

METHODS

Rats of the Wistar strain were employed. DAO activity was measured manometrically, employing D,L-alanine as the substrate (*3*). The liver was fractionated into nuclear, large granule (or mitochondrial) and supernatant fractions by differential centrifugation in 0.88 *M* sucrose (*4*). The adrenalectomized rats were maintained with salt (0.9 per cent NaCl) in the drinking water. Only those adrenalectomized animals which lost weight when the salt was omitted from the drinking water were used.

RESULTS

The livers of newborn rats were found to possess *ca.* 15 per cent of the DAO activity of adult rat liver (Fig. 1). The data obtained on young rats of various ages (and weights) are plotted on Fig. 1 as a function of the body weight of the rat. It can

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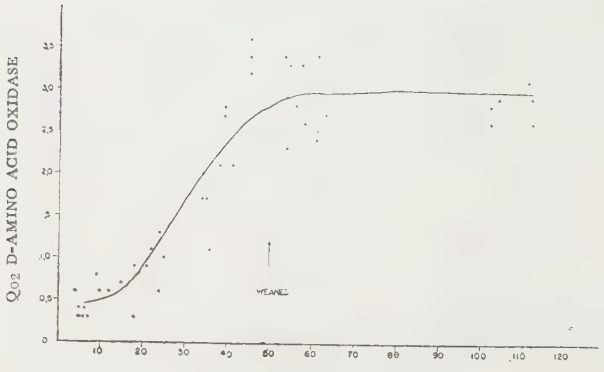
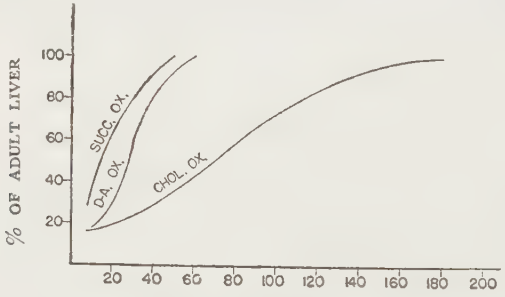
D-AMINO ACID OXIDASE ACTIVITY OF RAT LIVER AS A FUNCTION OF BODY WEIGHT



be seen that the activity of DAO increases rapidly to the adult level, attaining it by weaning time (*ca.* 21 days) and at a body weight of about 50 g.

This increase in activity is slightly slower than that of succinoxidase activity but much faster than choline oxidase activity (*5*).

ENZYMATIC ACTIVITY OF RAT LIVER AS A FUNCTION OF BODY WEIGHT



On the basis of available evidence (*6, 7, 8*) it was thought that all three enzymes were localized in the large granule fraction of the liver cells. However, cell fractionation studies on the DAO of normal rat liver indicated that this enzyme was equally distributed between the large granule fraction and the supernatant. These data are shown in Table I. The values shown are averages for 19 experiments. The recovery of DAO activity among the three fractions was 93 per cent. The distribution between the mitochondrial fraction and the supernatant varied markedly from animal to animal. In contrast, *ca.* 70 per cent of the choline oxidase activity in the livers of these animals was uniformly recovered in the mitochondrial fraction.

In view of the appreciable activity in both the mitochondrial and supernatant fractions, a group of 14 adrenalectomized rats was studied. Eighty-nine per cent of

TABLE I

Intracellular Distribution (in per cent) of the D-Amino Acid Oxidase

	No. of expt.	N	M	S
Control	19	8±1.7 (0.7<p<0.6)	43±3.4 (0.1<p<0.05)	46±3.7 (0.02<p<0.01)
Adrenalectomized	14	7±1.7	36±1.9	58±2.1

(N, M and S indicate nuclear, mitochondrial and supernatant fractions, respectively)

the total activity was recovered in the three fractions. As is shown in Table I, a larger percentage of the activity was recovered in the supernatant fraction and a smaller percentage in the mitochondrial fraction in the adrenalectomized rats. A calculation of the data using the T-test has shown that these differences are statistically significant.

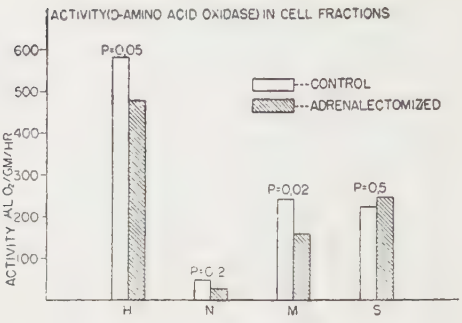
In contrast, no difference was noted in the distribution of choline oxidase activity in these livers and, as is indicated on Table II, this conclusion is also confirmed by statistical analysis.

TABLE II

Intracellular Distribution (in per cent) of the Cholibe Oxidase

	No. of expt.	N	M	S
Control	7	20±3.4 (0.3<p<0.2)	70±4.0 (0.6<p<0.5)	11±2.4 (0.7<p<0.6)
Adrenalectomized	6	13±4.5	74±3.7	13±3.2

If the data are expressed on an activity basis rather than on a per cent distribution basis as is shown in Fig. 3, it is seen that the activity of the DAO was significantly lower in the adrenalectomized rats than in the controls. The decrease in activity was not as great as that observed by Umbreit and Tonhazy (*2*) and averaged 18 per cent. Although somewhat less activity was recovered in the nuclear fraction, this difference was not significant. This was also true for the activity in the supernatant. However, there was a 35 per cent decrease in activity in the mitochondrial fraction. This difference is statistically significant. It would thus appear that in the livers of adrenalectomized rats the decrease in DAO activity occurs almost entirely in that portion of the activity associated with the mitochondrial fraction. The observation that the distribution of choline oxidase in the adrenalectomized livers was not altered suggests that the loss of DAO in the mitochondrial fraction may be a specific effect of adrenalectomy. However, the determination of the nitrogen content of the mitochondrial and supernatant fractions show that 12 per cent more nitrogen was found



in the supernatant and 10 per cent less nitrogen in the mitochondrial fraction from the adrenalectomized animals. This would indicate that there is a shift in protein from the mitochondrial to the supernatant fraction in the adrenalectomized rats. Whether this occurs in *in vitro* or was produced *in vitro* during homogenization is unknown. Preliminary measurements of the distribution of succinoxidase activity indicate that a larger proportion of this enzyme is also found in the supernatant fraction, although the activity of succinoxidase in the whole liver was not altered by adrenalectomy. These data indicate that the DAO activity in the large granule fraction is not the only enzyme decreased in this fraction and taken together with the nitrogen data suggest that the apparent selective loss of the DAO from the mitochondrial fraction may be explainable on the basis of increased friability of the large granules of adrenalectomyzed livers or due to changes in some of the large granules which make them less readily sedimentable.

SUMMARY

1. The D-amino acid oxidase activity of the livers of newbon Wistar strain rats is about 15 per cent of the adult liver level. The rate of increase is slightly slower than that of succinoxidase but much faster than choline oxidase.

2. In normal rat liver the D-amino acid oxidase is approximately equally distributed between the mitochondrial and supernatant fractions with less than 10 per cent of the activity associated with the nuclear fraction.

3. In adrenalectomized rats the D-amino acid oxidase activity was reduced by about 20 per cent, and this loss occurred in the mitochondrial fraction.

4. Adrenalectomy caused no marked change in choline oxidase activity of rat liver, nor any alteration of its distribution pattern.

REFERENCES

(*1*) Klein, J. R., *J. Biol. Chem.*, **128**, 650 (1939)
(*2*) Umbreit, W. W., and Tonhazy, N. E., *Arch. Biochem. Biophys*, **32**, 96 (1951)
(*3*) Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **134**, 749 (1940)
(*4*) Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948)
(*5*) Kensler, C. J., Rudden, M., Shapiro, E., and Langemann, H., *Proc. Soc. Exptl. Biol. Med.*, **79**, 39 (1952)
(*6*) Schneider, W. C., Claude, A., and Hogeboom, G. H., *J. Biol. Chem.*, **172**, 451 (1948)
(*7*) Claude, A., A. A. A. S. Research Conf. on Cancer, Washington, U. S. A., p. 223 (1945)
(*8*) Kensler, C. J., and Langemann, H., *J. Biol. Chem.*, **192**, 551 (1951)

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ÜBER PFLANZENPROTEASEN

IV.[1] EIN BEITRAG ZUR KENNTINIS DER BAKTERIENPROTEINASE

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Seitdem L. Geret und M. Hahn (*2*) zum ersten Male das sogenannte „Endo-Trypsin" in den Presssaften einiger Bakterien entdeckt haben, wurden mehrere Untersuchungen in diesem Gebiete durchgeführt. Auf Grund dieser Ergebnisse kann man heute annehmen, dass das proteolytische Enzymsystem im Bakterienleib aus Proteinase, Carboxypolypeptidase, Aminopolypeptidase, Dipeptidase und Acylase besteht (*3*). Trotzdem ist es bisher noch nicht gelungen, diese einzelnen Enzymkomponenten voneinander abgetrennt zu gewinnen. Im Anschluss an den schon früher von uns (*1*) beim Samenpulver und Algenthallus dargelegten Tatbeständen, haben wir nun die Autolysenmethode für die Bakterienmasse von *Staphylococcus aureus* und *Bacillus natto* aufgestellt, um damit die Proteinase von anderen Peptidasen zu befreien. Als Ausgangsmaterial diente das nach Buchnerscher Vorschrift (*4*) dargestellte Aceton-Dauerpräparat. Es wurde zur dreiwöchentlichen Autolyse unterworfen, das Digestat wurde dialysiert, und aus dem Dialysat liess ich das Enzym unter Verwendung des Acetons als farbloses Pulver darstellen. Die proteolytische Aktivität dieser Enzympräparate wurde nach der Methode von W. Grassmann u. H. Dyckerhoff (*5*) bestimmt, mit dem Ergebnis, dass 50 mg. *Staphylococcus*-Enzym zu 0.65 Hefe-Pr.-E., und 50 mg. *Natto*-Enzym zu 0.95 Hefe-Pr.-E. entsprechen.

Die auf diese Weise dargestellten Enzympräparate geben bei der Biuretprobe eine positive Reaktion mit rosarötlicher Farbe. Sie werden weder durch Erhitzung noch durch Sättigung mit Ammoniumsulfat ausgefallen. Hierbei sei es noch bemerkt, dass diese Enzympräparate mit Eosin fast quantitativ, mit Congorot sehr gut, mit Safranin order Fuchsin in geringem Masse ausgefallen werden, während sie mit Kry-

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stallviolett, Methylgrün, Neutralrot oder Pyronin gar nicht niedergeschlagen werden. Diese Tatsache erscheint auf ihre Basopepton-Natur hinzudeuten.

Um weiter in die enzymatische Natur dieser Enzympräparate Einsicht zu gewinnen, haven wir die Gelatinespaltung hinsichtlich der pH-abhängigkeit und HCN-Aktivierbarkeit studiert. Die Versuche ergaben, dass die Galatine sowohl durch *Staphylococcus*-Enzym als auch durch *Natto*-Enzym bei pH 5.0–6.0 optimal aufgespalten wird, und dass dabei der Zusatz von Cyanhydrin fast ohne Einfluss ist. Zur Bestimmung der enzymatischen Spezifität des *Natto*-Enzyms haben wir mit Glycyl-DL-asparagin, DL-Leucylglycylglycin, Benzoylglycin, Benzoylglycylglycin und Eisenin auf ihre Spaltbarkeit geprüft. Unter diesen Substanzen wurde das Eisenin aus *Eisenia bicyclis* dargestellt, und von uns (*6*) als Glutaminylglutaminsäureanhydrid-alanin erkannt. Beim Spaltungsversuche hat es sich ergeben, dass die gewöhnlichen Polypeptide durch dies Enzym gar nicht angegriffen werden, wohl aber das Eisenin ein gutes Substrat darstellt.

Hiermit sei es noch bezüglich der Angabe von M. J. Mycek *et al.* (*7*) zu erwähnen. Diese Autoren haben in letzterer Zeit eine Proteinase aus dem Kulturfiltrat von *Streptococcus* in krystallisiertem Zustande dargestellt, und deren enzymatische Spezifität mit verschiedenen Polypeptiden bestimmt und gefunden, dass dies Enzym erst nach Cysteinaktivierung Benzoyl-L-argininamid, Benzoyl-L-lysinamid, Carbobenzoxy-L-isoglutamin, Carbobenzoxy-L-isoasparagin und Benzoyl-L-histidinamid aufspalten vermag. Nach diesem Ergebnis haben sie darauf geschlossen, dass die Angreifbarkeit der Proteine durch Proteinasen im allgemeinen ausschliesslich durch die Art der seitlichen Atomgruppen der in Proteinen als solche vorhandenen Polypeptidketten bestimmt sei. Aber bei Erwägung des Umstandes, dass die Eiweissspaltung durch Bakterienproteinase in Abwesenheit jeglicher Aktivatoren zustande kommt, liegt uns der Gedanke nahe, dass die peptidatischen Wirkungen ihres Enzyms nichts mit dem ersten Angriff auf genuine Eiweisskörper zu tun haben. Wenn auch dem sein mag, doch bleibt nun natürlich die Frage offen gelassen, warum und wie sich das Protein ohne Zersprengung der in ihm innegehaltenen Peptidbindungen doch gegen Proteinasen zuständengemäss variierend verhalten könne. Diesbezügliche Tatsachen wurden schon früher von uns (*8*) beim Seidenfibroin, Ovalbumin und Enzymproteinen wie Pepsin, Trypsin und Papain dargetan.

Im Hinblick auf die oben dargestellten Erfahrungen haben wir nun

uns die Aufgabe angestellt, den Einfluss der Hitze-Denaturierung der Proteine auf Spaltbarkeit durch *Natto*-Proteinase näher zu verfolgen. Als Substrate dienten Casein, Glycinin, Hämoglobin, Ovalbumin, Serumalbumin, Legumelin und Ricin. Unter diesen Proteinen wurden das Glycinin und Legumelin aus Sojabohnen, und das Ricin aus Ricinussamen nach T. B. Osborne *et al.* (*9*) dargestellt, und das Hämoglobin wurde aus Rinderblut nach R. M. Ferry (*10*) in krystallisiertem Zustande gewonnen. Die Spaltungsversuche ergaben, dass Casein und Glycinin, ungekochtes oder gekochtes, durch *Natto*-Proteinase in gleichem Masse gut verdaut werden. Aber die Sache verhält sich etwas anders beim Hämoglobin, Ovalbumin, Serumalbumin, Legumelin und Ricin. Zwar diese Proteine sind alle in nativem Zustande gegen den Angriff der Bakterienproteinase sehr refraktär, während sie nach Denaturierung in leicht Spaltbares verändert werden.

An dieser Stelle möchten wir berechtigt sein, weiter zu gehen und zu fragen, was für Eiweissformen des Protoplasmas in lebenden Bakterienzellen der grossen Widerstandsfähigkeit gegen Autolyse zugrunde liegen. Um dieser Frage Aufschluss zu geben, haben wir somit nun unter Verwendung der Acetonpräparate von *Staphylococcus aureus* und *Bacillus natto* die autolytische Erscheinung bei verschiedenen Aciditäten zum näheren Studium herangezogen. Dabei wurde es festgestellt, dass das Plasmaprotein durch die intracellulären Proteasen unter Freisetzung äquivalenter Menge von COOH- bzw. NH_2-Gruppen bei pH 6.6–7.4 optimal aufgespalten wird. Sollte man den dabei freigemachten NH_2-N in Prozenten des Gesamt-(-CO-NH-)-N als Spaltungsgrad angeben, so ergab die autolytische Spaltung nach 3 Tagen im opt. pH-Bereiche bei *Staphylococcus aureus* zu 30.5% und bei *Bacillus Natto* zu 34.0%. Bezüglich des Umstandes, dass die Gelatinespaltung durch gereinigte Proteinase beim pH-Bereiche 5.0–6.0 optimal verläuft, während die Autolyse beinache in neutraler Reaktion optimal vonstatten geht, kann man annehmen, dass bei der Autolyse die in alkalischem Medium am stärksten wirksamen Peptidasen mitwirken. Aber in Berücksichtigung darauf, dass die Spaltung nicht so tiefer erreicht wird, scheint es hervorzugehen, dass die Peptidasen mit dem Altern rasch verloren gehen.

Bei dieser Gelegenheit sei noch darauf hingewiesen, dass die Bakterienzellen, in lebendem Zustande mit Farbstoffen schwer angefärbt werden, wohl aber sie nach Fixierung in gut Färbbares umgewandelt werden. Hierüber ist auch der Befund von R. Albert und W. Albert (*11*) beachtenswert, dass sich das mit Gentianaviolett färbbares Plasma-

protein in Hefezellen bei der Autolyse schnell verdaubar erweist. In Anbetracht dieser Sachlage haben wir weiter die Autolyse des Aceton-Dauerpräparates von *Bacillus natto* mit besonderer Rücksicht auf ihre Beeinflussbarkeit durch Farbstoffzusatz zur Ausführung gebracht. Die hierbei benutzten Farbstoffe sind Fuchsin, Krystallviolett, Methylgrün, Safranin, Neutralrot, Methylenblau, Thioninblau, Pyronin, Congorot, Carmin und Eosin. Um die Spaltbarkeit des Plasmaproteins in relativer Grösse auszudrücken, wurde der bei jedem Versuche freigemachte NH_2-N in Prozenten des maximalen Wertes, d. h. des bei ungefärbtem Präparat ergebenen, als relativer Spaltungsgrad angegeben. Dabei hat es sich herausgestellt, dass das Plasmaprotein der Bakterienzellen durch die eignen Proteasen sukzessiv schwerer beim Zusatz von Methylenblau, Methylgrün und Pyronin mit dem relativen Spaltungsgrad 76.3–85.2%, beim Zusatz von Congorot, Carmin, Krystallviolett und Thioninblau mit dem 62.8–69.7%, beim Zusatz von Safranin und Neutralrot mit dem 44.7–58.3%, und beim Zusatz von Eosin mit dem 28.3% aufgespalten.

Zieht man den Umstand, dass die Bakterienproteinase durch die sauren Farbstoff wie Eosin oder Congorot glatt abgefallen werden kann, und dies Enzym spezifisch für den Angriff des sauren Diketopiperazins eingestellt ist, in Betracht, so würde man nicht fehlgehen, auszusagen, dass die substratbindenden Basogruppen des Enzymproteins durch die sauren Farbstoffe teilweise besetzt werde, und dass die enzymaffinen Acidogruppen des Substratproteins durch die basischen Farbstoffe vor der Enzymwirkung partiell abgesperrt werden. Von diesem Gesichtspunkte aus kann das enzymologische Besonderheit des Plasmaproteins sodann dadurch verständlich gemacht werden, dass die sauren Atomgruppen dieses Proteins in lebenden Zellen in bezug auf dem Netzgebilde aus Diketopiperazinringen oder desgleichen stark geknäuelten Polypeptidketten in der Molekularoberfläche inwendig wirkungslos angelagert sind, und erst bei der Denaturierung vom Innere in die Oberfläche verlagert werden, um damit das Ringgebilde der Proteinasewirkung zugänglich machen zu lassen.

Hierbei sei es uns erlaubt, Herrn Prof. Dr. M. Ishidate für seine wohlwollende Unterstützung unseren wärmsten Dank auszusprechen. Die Ausführung dieser Arbeit wurde zum Teil durch das von Unterrichtsministerium dem einen von uns (Y. T.) gewährte Stipendium ermöglicht, wofür wir zum besten Dank verpflichtet sind.

EXPERIMENTELLES

I. Darstellung der Enzympräparate

(1) *Staphylococcus-Enzym—Staphylococcus aureus* wurde auf Bouillon-Agar bei 37° 7 Tage kultiviert. Der gewachsene Bakterienleib wurde mit Spatel gesammelt, und in kaltem Aceton eingetragen. Nach zweimaligem Wechsel des Acetons wurde die Bakterienmasse auf Nutsche filtriert, mit Äther nachgewaschen, und im Exsikkator über Schwefelsäure getrocknet. H_2O-Gehalt: 8.91%, Gesamt-N (Mikro-Kjeldahl): 6.20%, Gesamt-(-CO-NH-)-N (H_2SO_4-Hydrolysat, van Slyke): 4.48%. 10 g. Aceton-Dauerpräparat wurden in 200 ccm. Wasser verteilt und unter Toluolzusatz autolysiert. Nach einer Woche wurde das Digestat abfiltriert. Der Rückstand wurde weiter zweimal je mit 200 ccm. Toluolwasser zugesetzt und eine Woche lang bei Zimmer-temperatur überlassen. Darnach wurde die Suspension filtriert. Das vereinigte Filtrat wurde im Cellophanmembran unter Toluolzusatz 3 Tage lang gegen Leitungswasser dialysiert. Die dialysierte Flüssigkeit wurde im Vakuum unter 40° bis auf etwa 30 ccm. eingeengt, und mit mehrfachem Volumen kalten Acetons versetzt. Der dabei ausgeschiedene Niederschlag wurde auf Glasfilter gesammelt, mit Alkohol und Äther nachgewaschen, und im Exsikkator über Schwefelsäure getrocknet. Ausbeute betrug etwa 0.25 g.

(2) *Natto-Enzym*—Aus *Natto* isoliertes *Bacillus natto* wurde in 1000 ccm. Nährlösung von folgender Zusammensetzung gezüchtet: 10 g. Glutaminsäure-Na, 10 g. Glucose, 100 ccm. Sojabohnendekokt (aus 30 g. Samen). Kultivierungsdauer 72 Stdn. Temp. 37°. Die Reaktion der Lösung betrug pH 6.8 am Anfang und pH 8.0 am Ende. Die Kultur wurde dann zentrifugiert. Die einmal mit Wasser gewaschene, abzentrifugierte Bakterienmasse wurde in mehrfachem Volumen kalten Acetons eingetragen, und das Aceton-Dauerpräparat wurde auf die wie oben beschriebene Weise dargestellt. Gesamt-N: 6.80%, Gesamt-(-CO-NH-)-N: 4.53%. 20 g. Aceton-Dauerpräparat von *Bacillus natto* wurden in 1000 ccm. Toluolwasser drei Wochen lang bei Zimmertemperatur überlassen. Das Autolysat wurde wie üblich dialysiert. Aus dem konzentrierten Dialysat liess sich das Enzym unter Verwendung des Acetons als ein weisses Pulver in einer Ausbeute von etwa 0.50 g. darastellen. Gesamt-N: 9.55%.

II. Spezifität der Bakterienproteinase

(1) *pH-Abhängigkeit der Gelatinespaltung*—Versuchsanstellung: 1 ccm. Ansatz enthielt 60 mg. Gelatine, 5 mg. Enzym nebst variierender Menge der verschiedenen Salze als Puffer oder Aktivator. Toluol als Antiseptikum. Versuchstemp. 30°. Einwirkungsdauer 24 Stdn. Bestimmungsmethode: Die COOH-Zunahme wurde durch Titration in Alkohol mit 0.025 *N*-KOH gemessen (12). Das pH wurde colorimetrisch bestimmt. Die Ergebnisse sind in Tabelle I und Fig. 1 zusammengestellt.

(2) *Spaltbarkeit der Polypeptide durch Natto-Proteinase*—Versuchsanstellung: 1 ccm. Ansatz enthielt 0.05 mM. Polypeptid, 10 mg. Enzym und verschiedene Menge des Ammoniaks als Puffer. Toluol als Antiseptikum. Versuchstemp. 30°. Einwirkungsdauer 24 Stdn. Bestimmungsmethode: Wie oben. Der Spaltungsgrad wurde in Prozenten des maximalen Wertes, berechnet aus der spaltbaren Bindung, d. h. 1 (-CO-NH-)-Bindung pro Mol. der Substrate gekennzeichnet. Die Ergebnisse sind in

Tabelle I

Zusatz mM. pro 1 ccm. Ansatz		pH der Lösung	COOH-Zunahme in ccm. 0.025 *N*-KOH	
			Staphyloc.-Enzym	*Natto*-Enzym
HCl	0.025	4.0	0.60	0.60
CH_3COOH	0.030	5.0	1.30	1.80
KCN	0.010	5.2	1.30	1.90
CH_3COOH	0.008			
—	—	6.0–5.6	1.20	1.65
NH_4OH	0.040	8.0–7.6	0.80	1.20
NH_4OH	0.100	9.0–8.6	0.50	0.80

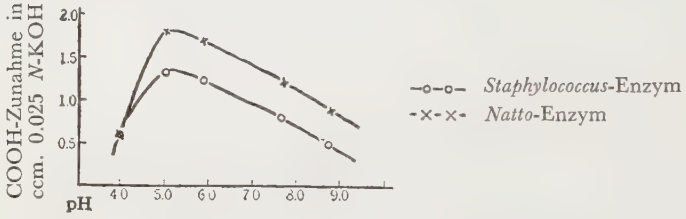

Fig. 1. pH-Abhängikeit der Gelatine-spaltung durch Bakterien-proteinase.

Tabelle II

Substrat	Zusatz als Puffer NH_4OH *mM.*	pH	COOH-Zunahme in ccm. 0.025 *N*-KOH	Spaltungs-grad (%)
Glycyl-DL-asparagin	0.05	3.2	0.00	0.0
DL-Leucylglycylglycin	0.025	7.0	0.00	0.0
Benzoylglycin	0.05	5.0	0.00	0.0
Benzoylglycylglycin	0.05	5.0	0.00	0.0
Eisenin	0.05	5.0	0.80	40.0

der Tabelle II angegeben.

(3) *Einfluss der Hitze-Denaturierung der Protein auf ihre Spaltbarkeit durch Natto-Proteinase*—Versuchsanstellung: 1 ccm. Ansatz enthielt 50 mg. Substratprotein und 5 mg. Enzym. Toluol als Antiseptikum. Temp. 30°. Einwirkungsdauer 24 Stdn. pH 5.4–6.0. Bestimmungsmethode: Wie üblich. Die Ergebnisse sind in Tabelle III angegeben.

Tabelle III

Substrat	COOH-Zunahme in ccm. 0.025 *N*-KOH	
	Ungekochtes Protein	Gekochtes Protein
Casein	1.40	1.40
Glycinin	0.90	0.90
Hämoglobin	0.20	0.90
Ovalbumin	0.10	0.80
Serumalbumin	0.10	0.35
Legumelin	0.00	1.10
Ricin	0.10	1.30

III. Autolyse der Aceton-Dauerpräparate der Bakterien

(1) *pH-Abhängigkeit der Autolyse*—Versuchsanstellung: In 5 ccm. Ansatz sind 250 mg. Acetonpräparat und verschiedene Menge der Salzsäure und des Ammoniaks als Puffer enthalten. Temp. 30°. Versuchsdauer 72 Stdn. Das Digestat wurde filtriert, und 1 ccm. Filtrat (entsprechend 50 mg. Sbst.) wurde zur Analyse herangezogen. Als Kontrolle dienten die anfangs gekochten Ansätze. Bestimmungsmethode: Die COOH-Zunahme wurde durch Titrationsmethode nach R. Willstätter u. E. Waldschmidt-Leitz (*12*) bestimmt, die NH_2-Zunahme wurde durch gasvolumetrische Methode nach van Slyke (*13*) gemessen, und diese Werte wurden in Prozenten des Gesamt-(CO-NH-)-N als Spaltungsgrad angegeben. Die Ergebnisse sind in Tabelle IV und Fig. 2 veranschaulicht.

Tabelle IV

Zusatz als Puffer pro 50 mg. Sbst. mM.	pH	*Staphyloc. aur.*		*Bacillus natto*		
		COOH-Zunahme in ccm. 0.025 *N* KOH	Spaltungsgrad (%)	COOH-Zunahme in ccm. 0.025 *N* KOH	NH_2-Zunahme in ccm. 0.025 mM. Aquiv.	Spaltungsgrad (%)
HCl 0.05	3.0	0.20	3.1	—	—	—
HCl 0.025	4.0–4.4	1.10	17.2	0.95	0.97	15.0
— —	6.0–5.6	1.80	28.1	1.80	1.76	27.2
NH_4OH 0.05	7.4–6.6	1.95	30.5	2.20	2.04	31.5
NH_4OH 0.10	8.4–7.8	1.55	24.2	1.70	1.72	26.6

(2) *Einfluss des Zusatzes von Farbstoffen auf die Autolyse*—Versuchsanstellung: 50 mg. Aceton-Dauerpräparat von *Bacillus natto* wurden in 1 ccm. Wasser oder Farbstofflösung (gewöhnlich 0.5%) verteilt. Die benutzten Farbstoffe sind alle Präparate von Grübler oder Merck. Beim Versuche mit Eosin, Carmin, Neutralot wurde die gesättigte wässrige Lösung zur Verwendung gebracht. Toluol als Antiseptikum. Temp. 30°. Versuchsdauer 144 Stdn. pH 5.6–6.2. Bestimmungsmethode: Das Digestat

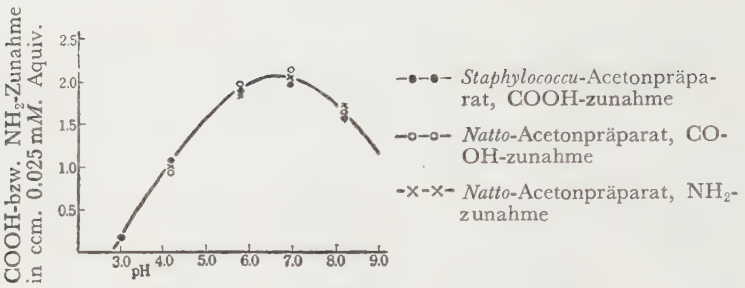

Fig. 2. pH-Abhangigkeit der Autolyse der Bakterien

wurde auf Glasfilter filtriert. Der Rückstand wurde mehrmals mit heissem Wasser nachgewaschen. Das Filtrat und Waschwasser wurden vereinigt, und diese Lösung wurde zur Analyse herangezogen. Als Kontrolle dienten die vorher gekochten Präparate. Zur Ermittelung der Autolysenstärke wurde der in jedem Versuche zugewachsene NH_2-N nach van Slyke bestimmt, und diese Werte in Prozenten vom Gesamt-(-CO-NH-)-N als Spaltungsgrad angegeben, und in Prozenten des maximalen Wertes, k. h. des beim Versuche ohne Farbstoffzusatz ergebenen, als relativer Spaltungsgrad gekennzeichnet. Die Ergebnisse sind in der folgenden Tabelle V zusammengestellt.

Tabelle V

Farbstoff	NH_2-N-Zunahme mg.	Spaltungsgrad (%)	Relativ. Spaltungsgrad (%)
—	0.600	25.5	100
Fuchsin	0.198	8.7	33.0
Krystallviolett	0.403	17.8	67.2
Methylgrün	0.511	22.5	85.2
Safranin	0.268	11.8	44.7
Neutralot	0.350	15.5	58.3
Methylenblau	0.458	20.2	76.3
Thioninblau	0.418	18.5	69.7
Pyronin	0.511	22.6	85.2
Congorot	0.377	16.6	62.8
Carmin	0.381	16.8	63.5
Eosin	0.171	7.5	28.5

ZUSAMMENFASSUNG

Unter Verwendung der Autolysenmethode wurde eine peptidasenfreie Proteinase aus Aceton-Dauerpräparaten von *Staphylococcus aureus* und *Bacillus natto* dargestellt. Die Spaltungsversuche mit Proteinen und Polypeptiden bei verschiedenem pH ergaben, dass die Bakterienpro-

teinase der Kathegorie von Papain oder Kathepsin angehört, und dass ihre enzymatische Spezifität in der Aufschliessung des sauren Diketopiperazins besteht. Weiter wurden die enzymatische Spaltbarkeit des Albumins tierischer oder pflanzlicher Herkunft einerseits, und die Autolysierbarkeit des Plasmaproteins in den Bakterienzellen andererseits, insbesondere hinsichtlich der Hitze-Denaturierung und der Färbbarkeit mit sauren und basischen Farbstoffen, näher studiert. Aus diesen Ergebnissen sind die Autoren zum Schluss gekommen, dass die gegensätzlich geladenen ionischen Atomgruppen an der Oberfläche des Enzym-bzw. Substrat-Moleküls sowohl bei der Proteolyse als auch bei der Autolyse eine dirigierende Rolle spielen.

LITERATUR

(*1*) III. Mitteilung: *Bot. Mag. Tokyo*, **66**, 77 (1953)
(*2*) Geret, L., und Hahn, M., *Ber. dtsh. chem. Ges.*, **31**, 2335 (1898); *Z. Biol.*, **40**, 117 (1900)
(*3*) Imaizumi, M., *J. Biochem.*, **27**, 45, 65, 199, 213 (1938)
(*4*) Albert, R., Buchner, E., und Rapp, R., *Ber. dtsch. Chem. Ges.*, **35**, 2376 (1902)
(*5*) Grassmann, W., und Dyckerhoff, H., *Z. physiol. Chem.*, **179**, 41 (1928)
(*6*) Tazawa, Y., *Acta Phytochim.*, **15**, 141 (1949)
(*7*) Mycek, M.J., Elliott, S.D., und Fruton, J.S., *J. Biol. Chem.*, **197**, 637 (1952)
(*8*) Tazawa, Y., *Acta Phytochim.*, **13**, 57, 117 (1942–3); **14**, 75 (1944); **15**, 73 (1949)
(*9*) Osborne, T.B. *et al. J. Am. Chem. Soc.*, **20**, 419 (1898); *Am. J. Physiol.*, **14**, 259 (1905)
(*10*) Ferry, R.M., *J. Biol. Chem.*, **57**, 819 (1923)
(*11*) Albert, R., und Albert, W., *Zentrabl. Bakt.* II. **7**, 737 (1901)
(*12*) Willstätter, R., und Waldschmidt-Leitz, E., *Ber. dtsh. chem. Ges.*, **54**, 2988 (1921); Grassmann, W., und Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929)
(*13*) Van Slyke, D.D., *J. Biol. Chem.*, **23**, 407 (1915)

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STUDIES ON XANTHURENIC ACID

VII. ON THE "XANTHURENICASE"

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Xanthurenic acid, first discovered by Musajo (*1*), has since been studied by Lepkovsky *et al.* (*2*), and identified to be an abnormal metabolite of tryptophane. It has recently been confirmed further by Kotake, Jr., Inada, *et al.* that an administration to a white rat of sodium butyrate together with tryptophane led to produce in its organism xanthurenic acid (*3*), which in turn gave rise to genuine diabetic symptoms (*4*, *5*).

Since fatty acid and tryptophane are common but important nutrients for us it is expected that xanthurenic acid may be somewhat produced in daily metabolic processes. The acid, however, is hardly found in normal urine, so that it might be assumed that xanthurenic acid may be decomposed by some process perhaps of enzymic nature. In this paper a study on the existence of such an enzyme is presented.

EXPERIMENTAL

For the determination of xanthurenic acid, the quantitative analysis method of Inouye and Nogami (*6*), which is the modification of Glazer's method (7) was employed. Namely, the test solution was deproteinized by the addition of sulfuric acid and sodium tungstate. The supernatant obtained was adjusted to pH value of 3.4–4.0. This supernatant and $FeNH_4(SO_4)_2$ solution were added to a 7 : 3 mixture of a saturated $NaHCO_3$ solution and a saturated Na_2CO_3 solution. The whole solution was neutralized by 1N HCl with phenolphtalein as indicator just to the point of disappearance of its pink colour. Then the remaining green colour due to xanthurenic acid was determined colorimetrically using filter S_{61} for photoelectric colorimetry.

Experiment I

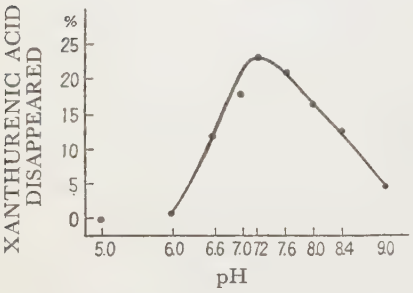
One ml. of watery extract corresponding to 1 g. of the liver tissue or of the kidney tissue of rabbit or of white rat was mixed with 3 ml. of phosphate buffer solution (pH 7.0) containing 4 mg. of xanthurenic acid and incubated at 38°. It turned out

that the longer the incubation, the more the admixed xanthurenic acid disappeared. This occurred most apparently with rabbit's liver tissue. Thus it was substantially proved that such watery extracts contained an enzyme capable of decomposing xanthurenic acid. The authors give the name "Xanthurenicase" to this enzyme. For further experiments the liver-tissue extracts of rabbit were used as the enzyme solution.

Experiment II

On the reaction mixture consisting of 1 ml. of the enzyme solution, 1 ml. of buffer solution and 2 ml. of phosphate buffer containing 4 mg. of xanthurenic acid were studied effects of pH, temperature, incubation time and inhibitor.

Fig. 1



Effect of pH—To bring about different pH between 6.0 and 9.0 phosphate buffer or NaOH-glycin buffer was used. The incubation time was 4-hour and the temperature 38°. The result is shown in Fig. 1. The pH optimum is situated at 7.2.

Effect of Temperature—The reaction mixture was regulated to pH 7.2. Then 3-hour incubation was conducted at different temperatures of 13°, 38°, and 50°, as shown in Table I. The disappearance of the admixed xanthurenic acid was much less at 13° and 50° than at 38°.

Table I

Temperature	Disappearance of the admixed xanthurenic acid
	%
13°	6.0
38°	19.7
50°	1.7

It was found further that when the enzyme solution had beforehand been heated for 15 minutes at 80°, the disappearance of the admixed xanthurenic acid was not noticeable even after 4-hour incubation at 38°.

Effect of Incubation Time—Next, under the condition of the optimal pH and temperature, it was examined how far the decomposition of xanthurenic acid proceeds with time. The result was illustrated in Fig. 2. It was found that about one third of the admixed xanthurenic acid disappeared in around 6 hours.

Effect of Inhibitors—To the reaction system was added respectively sodium fluoride

up to *M*/100, monoiodoacetic acid up to *M*/200 or potassium cyanide up to *M*/500 or *M*/1000 concentration. The incubation time was 5 hours, pH 7.2 and the temperature 38°. As Table II shows, the disappearance of the admixed xanthurenic acid was least inhibited in the case of *M*/1000 KCN, while in the cases of *M*/500 KCN, *M*/100 NaF and *M*/200 CH_2ICOOH, the inhibition was exceedingly remarkable.

FIG. 2

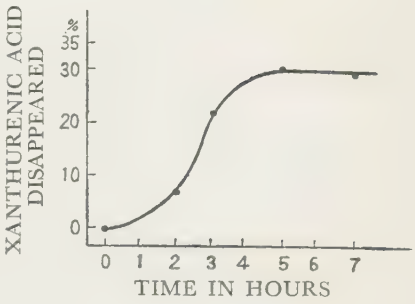


TABLE II

	Concentration Inhibitor	Disappearance of the admixed xanthurenic acid	The rate of inhibition
	None (Control)	33.7%	%
M/ 500	KCN	14.7	56
M/1000		29.0	14
M/ 100	NaF	10.5	69
M/ 200	CH_2ICOOH	11.4	66

Experiment III. Trials to Purify the Enzyme Fractionation with Saturated $(NH_4)_2SO_4$

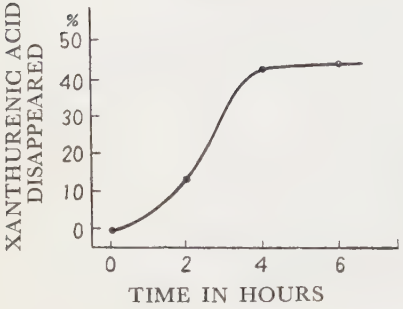
In order to obtain a pnre enzyme, fractionation with ammonium sulfate was attempted.

a) The precipitate obtained by 50 per cent saturation with ammonium sulfate turned out to be a globulin fraction, while the precipitate obtained from the supernatant by 100 per cent saturation by adding solid $(NH_4)_2SO_4$ proved to be an albumin fraction. These two different fractions were tested for the enzyme activity. The result was that the disappearance of the admixed xanthurenic acid was not noticeable in the case of the albumin fraction but in the case of the globulin fraction. An example of the results obtained by 4-hour incubation is shown in Table III.

TABLE III

	Disappearance of the admixed xanthurenic acid (pH 7.2, 38°)
	%
Albumin fraction	0.1
Globulin fraction	34.2

Fig. 3



by 4 hours incubation.

b) By employing the globulin fractions, effects of time were examined. In around 4 hours 30–50 per cent of the substrate disappeared. An example is shown in Fig. 3.

c) The globulin portion was further fractionated and each fraction was examined. The maximal enzyme action was noticeable when 20 per cent or thereabouts saturation concentration of ammonium sulfate was reached. Table IV shows an example of the results obtained

Table IV

Fraction	Disappearance of the admixed xanthurenic acid
0—10% saturation	1.4%
10—20 ,, ,,	41.6
20—30 ,, ,,	16.0
30—40 ,, ,,	6.8
40—50 ,, ,,	0.8

d) Fractions obtained at 10–20 per cent saturation concentrations of ammonium sulfate were taken up and the effect of time was examined. In around one and a half hour of incubation, 30–50 per cent disappearance of the substrate could be noticed. An example is shown in Fig. 4.

Experiment IV. Adsorption Test

This time enzyme purification was attempted by means of adsorption. It turned out that the enzyme in question could never be adsorbed by Cγ but that it could best be adsorpted by Kaolin and eluted by sodium carbonate. The details of the procedure as shown in Table V.

The enzyme activity was tested on the reaction mixture of the eluate corresponding to 2 g. of rabbit liver and phosphate buffer containing 8 mg. of xanthurenic acid, the total volume being 8 ml. The result is illustrated in Fig. 5.

Experiment V. Preparation of Desiccated Powder of A Rabbit Liver Tissue

Desiccated powder of a rabbit's liver tissue was prepared by the method shown

in Table VI.

Fig. 4.

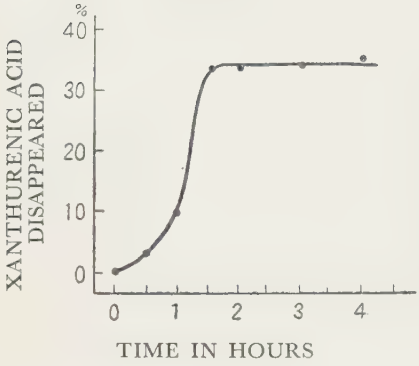


Fig. 5

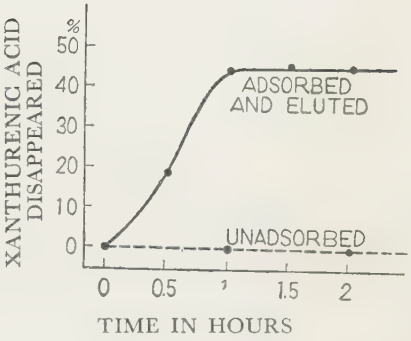


Table V

The extract of a rabbit's liver tissue

10% saturation with $(NH_4)_2SO_4$

Supernatant — Precipitate (discarded)

30% saturation with $(NH_4)_2SO_4$

Precipitate — Supernatant (discarded)

Adjust to pH 5.2–5.6 by $M/15$ KH_2PO_4; shake up for 30 minutes after the addition of Kaolin in 1/10 volume; wash the precipitate with the solution (pH 5.2–5.6)

Precipitate — Supernatant (unadsorbed)

Adjust to pH value 8.2–8.4 by Na_2CO_3 solution; shake up for 30 minutes; wash the precipitate with the solution (pH 8.2–8.4)

Eluate — Precipitate (discarded)

A portion of the powder was mixed with ten times as much 87 per cent glycerol or water and after shaking up, left at room temperature for 24 or 12 hours. The supernatant was separated by centrifuge, on which enzyme activity was determined as usual.

An example is shown in Fig. 6.

Table VI

From fresh rabbit liver tissue remove fat and fibre as much as possible, and mince into paste.

A g.

↓ Mix with 2×A ml. of acetone; filter after shaking up for one hour.

Residue

↓ Mix with 2×A ml. of acetone; filter after shaking up for one hour.

Residue

↓ Mix with 2×A ml. of an acetone-ether mixture (1 : 1); filter after shaking up for one hour.

Residue

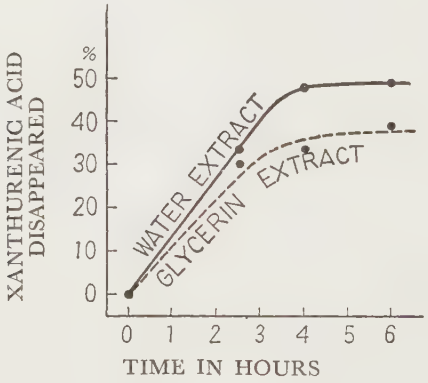
↓ Mix with 2×A ml. of ether; filter after shaking up for one hour.

Residue

↓ Crush and spread on a sheet of filter paper for airing; pulverize in a motar and sift by the Japanese pharmaceutical No. 6 sieve.

Desiccated powder (Total yield approximately (1/6) A g.)

Fig. 6



Experiment VI. Paper Partition Chromatographic Test

In order to ascertain the existence of a certain product of xanthurenic acid by the section of the enzyme paper-partition-chromatography was applied. The results are illustrated in Fig. 7.

The spots were detected fluorescopically and also identified by various color reactions as shown in Table VII. It is clear that F4 F3′ and F1‴ are due to xanthurenic

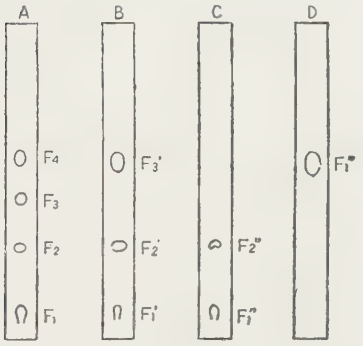


Fig. 7. Experimental conditions: Aqueous extract of a rabbit's desiccated liver-tissue powder was employed for the enzyme solution. The reaction mixtures were as follows.

A→Enzyme solution+phosphate buffer solution (pH 7.2)+substrate

B→Enzyme solution+phosphate buffer solution and substrate were incubated separately and mixed just before the paper-partition-chromatographic test.

C→Enzyme solution+phosphate buffer solution

D→Substrate+phosphate buffer solution

After being incubated for 5 hours at 38°, the reaction mixture was extracted with as much alcohol. On this alcoholic extract paper-partition-chromatography was applied using No. 52 "Toyo" filter paper and a developer of methanol, *n*-butanol, benzene and water (4:2:2:2).

acid. F3 is a spot due to a reactions product of xanthurenic acid by the enzyme.

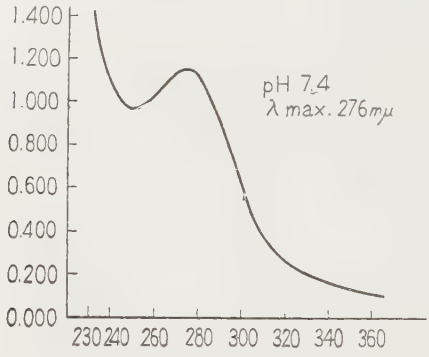
Table VII

Color reaction	A				B			C		D
	F1	F2	F3	F4	F1′	F2′	F3′	F1″	F2″	F1‴
Rf value	0.09	0.28	0.43	0.56	0.09	0.30	0.57	0.09	0.30	0.57
Ehrlich	−	−	−	+	−	−	+	−	−	+
Pauly	−	−	−	+	−	−	+	−	−	+
Millon	−	−	−	+	−	−	+	−	−	+
Fe^{+++}	−	−	−	+	−	−	+	−	−	+
Ninhydrin	−	−	−	−	−	−	−	−	−	−
Bratton-Marshall	−	−	−	−	−	−	−	−	−	−
Reduction	+	−	−	+	+	−	+	+	−	+

Experiment VII. Absorption Spectrum Test

To get some clue for the elucidation of chemical nature of the reaction product of xanthurenic acid, the spots (F_3) were cut out and collected. Absorption spectrum was taken of the substance responsible for the spot F3 in the foregoing experiment. Namely, the portions of several sheets of filter paper marking the spot F3 were cut out and subjected to extraction in phosphate buffer solution (pH 7.4). Then the extract was redeveloped by Levy and Chung's method (*8*). In this case water-saturated butanol was used as a developer and filter paper No. 52 "Toyo", which was previously sprayed with a mixture (pH 9.3) of *M*/10 boric acid and *N*/10 NaOH. After 15 hours of development the paper was dried and the spots were detected fluorescopically. The spots (Rf=0.40) thus found were cut out and subjected again to extraction in phosphate buffer solution (pH 7.4). On this extract the ultraviolet spectrum was examined by Beckman photometer, and the result shown in Fig. 8 was obtained.

FIG. 8



The results of colorimetric and spectrophotometric investigations indicate that the decomposition of xanthurenic acid by the enzyme is fairly drastic. Perhaps pyridine ring is split up and some phenol derivative is produced.

SUMMARY

1. It was found that three existed an enzyme in liver tissue capable of decomposing xanthurenic acid. The name, "Xanthurenicase", was given to this enzyme.

2. Several characteristics of this enzyme such as pH optimum, temperature optimum, inhibitor, etc. were studied.

3. Based on the examinations by both paper-partition-chromatography and absorption spectrum, some postulation is presented that the substance formed by the decomposition of xanthurenic acid may be

a derivate of phenol caused by the splitting of a pyridin ring of xanthurenic acid.

REFERENCES

(*1*) Musajo, L., Atti accad. *Lincei*, **21**, 368 (1935)
(*2*) Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943)
(*3*) Kotake, Y. Jr., and Inada, T., *J. Biochem.*, **40**, 287 (1953)
(*4*) Kotake, Y. Jr., and Inada, T., *J. Biochem.*, **40**, 291 (1953)
(*5*) Inada, T., Matsumura, Y., and Inouye, G., *J. Wakayama Med. Soc.*, **4**, 114 (1953)
(*6*) Inouye, A., and Nogami, K., *J. Wakayama Med. Soc.*, **4**, 125 (1953)
(*7*) Glazer, H. S., Mueller, J. F., Thompson, C., Hawkins, V. R., and Vilter, R. W., *Arch. Biochem. Biophys.*, **33**, 243 (1951)
(*8*) Levy, A. L., and Chung, D., *Anal. Chem.*, **25**, 396 (1953)

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STUDIES ON XANTHURENIC ACID

VIII. INTERACTIONS BETWEEN XANTHURENIC ACID AND ACETONE BODIES IN RAT

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(Received for publication, February 24, 1954)

As Kotake and Inada (*1*) reported in their preceding paper, tryptophane, when administered to a white rat simultaneouly with a fairly large dose of sodium butyrate, causes an abnormal metabolism, producing xanthurenic acid in its organism and thereby developing diabetic symptoms. It is possible to consider in this connection that such symptoms are closely correlated to the functions of the liver, and in order to ascertain it further, we have been conducting a series of experiments on the probable interactions between xanthurenic acid and acetone bodies often detected in cases of weakened liver-functions, especially in those of severe diabetic symptoms. The results hitherto obtained are reported here.

Along with the above, another series of experiments have been made on the effects of anthuranilic acid upon acetone bodies (*2*) and of pyridoxine. These experimental results are also reported in the present paper.

MATERIALS AND METHODS

Materials—

White rats weighing from 150 to 200 g.

Pure synthetic xanthurenic acid the melting point of which is 295.5–296°.

Quantitative Analysis—

The total amount of acetone bodies contained in the urine of each rat was determined by the method of Borchers, Berg and Whitmann (*3*) which is a modification of Van Slyke's method. By this modification kynurenine and other hindering substances could be eliminated.

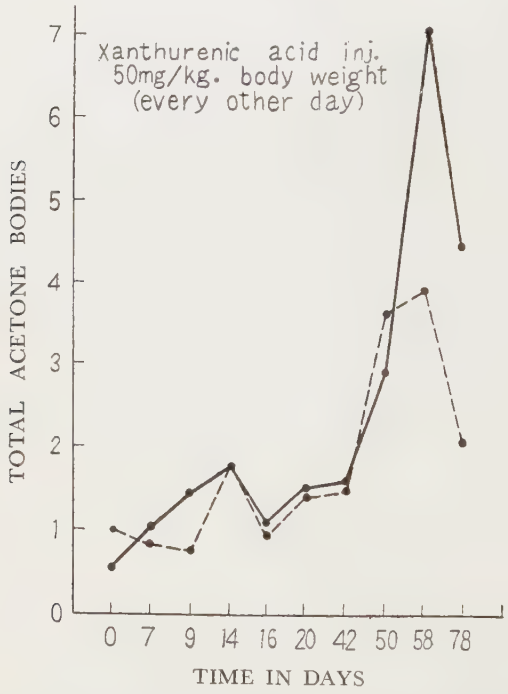
For the determination of the xanthurenic acid, the method established by Glazer *et al.* (*4*) was used and for the determination of the blood sugar the analytical method worked out by King and Garner (*5*).

RESULTS

I. Effect of Xanthurenic Acid upon the Formation of Acetone Bodies in the Body

An injection of 50 mg. of xanthurenic acid per kg. of each white rat weighing about 150 g. was repeated every other day and the total amount of acetone bodies in the 24 hour's urine was determined. The result was that following each injection, acetone bodies gradually increased in quantity until about seven times as much as the value obtained before the injection (see Fig. 1).

FIG. 1. (two examples)



Next, white rats were fed for a length of time both on casein diet rich in fat and on casein diet deficient in vitamin B_6 as shown in Tables I and II, respectively. These diets were proved to give rise to the production of a large amount of xanthurenic acid in rat as reported already by Kotake, Inada and Matsumura (*6*) in their preceding paper. In both cases a remarkable increase in acetone bodies by nearly ten times (Figs. 2 and 3) was observed. There is little doubt that the

TABLE I

Casein Diet Containing Rich Fat	
Casein	25%
McCollum salt mixture	2%
Agar	3%
Butter	35%
Sucrose	5%
Yeast	2%
Starch	28%

TABLE II

Casein Diet Deficient in Vitamin B_6	
Casein (Hammarsten)	20%
Corn oil + halibut liver oil	4%
McCollum salt mixture	2%
Sucrose	74%
Vitamin B_1	3.3 γ/g.
Nicotinic acid	10.0 ,,
Calcium pantothenate	13.3 ,,
Riboflavin	6.6 ,,
Choline chloride	166.0 ,,
Inositol	333.0 ,,
p-Aminobenzoic acid	200.0 ,,

xanthurenic acid produced in the body of rat was responsible for such an increase in acetone bodies.

II. Effects of Anthranilic Acid and Pyridoxine upon the Formation of Acetone Bodies in Rat

Eight white rats, each weighing about 170 g., were employed. To four of them a twice-a-day administration of 0.4 g. of sodium butyrate was continued for three successive days, which caused of course abundant excretion of acetone bodies into urine. For a week thereafter no sodium butyrate administration was conducted. When the amount of acetone bodies in the urine returned to normal, the former daily sodium butyrate administration was resumed, and this time a daily injection of 1 mg. of anthranilic acid for three days was executed.

With the remaining four rats the same treatments as stated above were conducted in adverse order. The injection of anthranilic acid in each case resulted in suppressing increase of acetone bodies to a great extent (see Table III).

But it should be noticed that too much anthranilic acid, for instance 10 mg. per day, caused rather contrary effect as shown in Table IV.

Next, the effect of pyridoxine was examined. Four rats were subjected to the daily injection of 2 mg. each of pyridoxine for three successive days. Again, a decrease in acetone bodies by half was observed as shown in Table V.

From the foregoing experiments we are led to believe that both anthranilic acid and pyridoxine are effective in checking the formation of acetone bodies in rat.

TABLE III

Anthranilic Acid 1 mg. per Day Injection+Na-butyrate 0.4 g. per Day per os.

No.	1st day	2nd day	3rd day	Total	Substances fed
1	13.7mg.	9.8mg.	25.9mg.	49.4mg.	Anth. acid+Na butyrate
	19.0	38.5	46.5	104.0	Na butyrate
2	14.0	18.7	12.4	45.1	Anth. acid+Na butyrate
	13.6	20.4	11.2	45.2	Na butyrate
3	5.0	10.9	18.5	34.4	Anth. acid+Na butyrate
	5.3	12.5	25.3	43.1	Na butyrate
4	3.5	10.2	12.8	26.4	Anth. acid+Na butyrate
	7.6	16.1	17.7	41.5	Na butyrate
5	3.8	7.8	11.6	23.2	Anth. acid+Na butyrate
	7.8	15.8	25.2	48.5	Na butyrate
6	6.3	15.4	6.7	28.4	Anth. acid+Na butyrate
	14.1	20.4	26.0	60.5	Na butyrate
7	11.6	10.8	14.2	36.6	Anth. acid+Na butyrate
	4.9	25.7	29.5	60.1	Na butyrate
8	6.1	4.7	7.3	18.1	Anth. acid+Na butyrate
	5.3	11.3	30.5	47.1	Na butyrate

Anth. acid=anthranilic acid. ♀ rats, body weight about 170 g.

FIG. 2. (two examples)

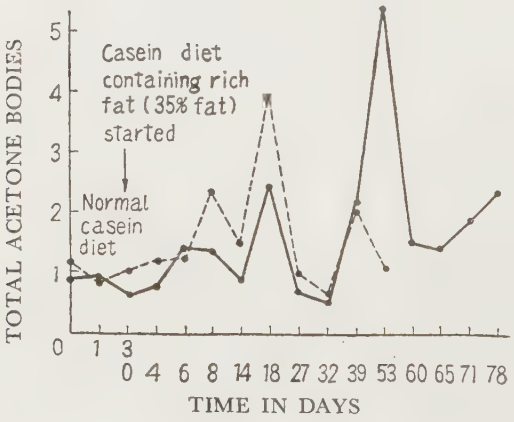


TABLE IV

Anthranilic Acid 10 mg. per Day Injection + Na-butyrate 0.4 g. per Day per os.

No.	1st day	2nd day	3rd day	Total	Substances fed
1	4.4 mg.	30.5 mg.	61.3 mg.	96.2 mg.	Anth. acid + Na butyrate
	2.6	21.3	18.0	41.9	Na butyrate
2	17.6	20.7	40.3	78.6	Anth. acid + Na butyrate
	3.6	10.5	11.3	25.0	Na butyrate
3	16.2	29.3	90.7	136.2	Anth. acid + Na butyrate
	15.1	14.1	85.6	115.3	Na butyrate
4	19.1	24.8	23.1	67.0	Anth. acid + Na butyrate
	13.0	20.3	24.9	58.2	Na butyrate
5	14.6	31.6	40.2	86.4	Anth. acid + Na butyrate
	12.7	23.4	22.0	58.1	Na butyrate

Anth. acid = anthranilic acid. ♀ rats, body weight about 150 g.

TABLE V

Pyridoxine 2 mg. per Day Injection + Na-butyrate 0.4 g. per Day per os.

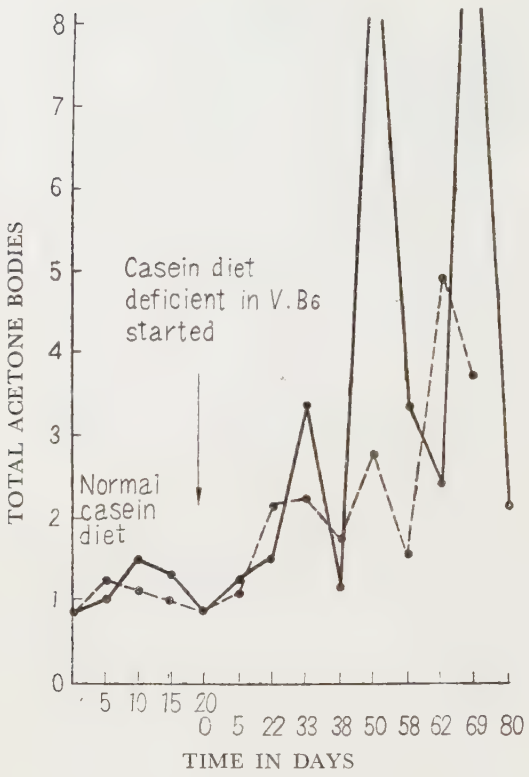
No.	1st day	2nd day	3rd day	Total	Substances fed
1	4.8 mg.	4.0 mg.	3.6 mg.	12.4 mg.	V. B_6 + Na butyrate
	9.7	8.9	5.6	24.2	Na butyrate
2	6.0	9.7	7.3	22.0	V. B_6 + Na butyrate
	7.1	14.8	24.2	46.1	Na butyrate
3	6.3	5.5	16.7	28.5	V. B_6 + Na butyrate
	13.0	14.1	13.5	40.6	Na butyrate
4	9.7	8.8	5.3	23.8	V. B_6 + Na butyrate
	20.3	20.9	30.7	75.5	Na butyrate

♀ rats, body weight about 150 g.

III. Effect of Acetoacetic Acid upon the Formation of Xanthurenic Acid in Rat

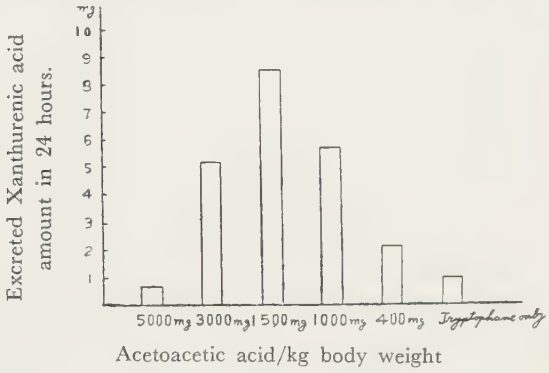
To a group of white rats fed on the afore-mentioned synthetic diet for more than a week, various quantities of acetoacetic acid were administered together with 100 mg. of tryptophane, and the amount of xanthurenic acid in the 24 hour's urine of each rat was determined. In this case, the excreted amount of xanthurenic acid was always larger than when acetoacetic acid was not administered. The largest

FIG. 3. (two examples)



quantity of xanthurenic acid was excreted in the urine—as shown in Fig. 4—when an administration of 1500 mg./kg. was conducted. Too much of acetoacetic acid deteriorates the functions of liver and tryptophane metabolism seems to cease, never going on beyond the stage of kynurenine. This is justified by the finding that paper partition chromatograms revealed the existence of kynurenine only. As to the behaviour of blood sugar in these cases it is interesting to note that it increased in parallel to the increase of xanthurenic acid. For instance the rat showed 64.2 mg./dl. blood sugar before the administration of acetoacetic acid, while it rose to 113.1 mg./dl. on the fourth day after the administration, both determined when the stomach was empty. Compared with the result of the previous experiment on the effect of tryptophane and sodium butyrate reported by Kotake and Inada (*1*), the rise of blood sugar was not so pronounced. This may possibly be attributed to the decrease of appetite of the rats caused by acetoacetic acid.

FIG. 4. Rats, ♂, weighing about 150 g. (Tryptophane 0.1 g. + acetoacetic acid *per os*.)



SUMMARY

1. The formation of xanthurenic acid and that of acetone bodies are closely correlated. Increased xanthurenic acid never fails to lead to increased out-put of acetone bodies. This fact is of no small interest and encourages us to further studies on diabetic symptoms of rats caused by xanthurenic acid.

2. It has been made clear that both a proper quantity of anthranilic acid and of pyridoxine are capable to check the formation of acetone bodies in rat.

We with to thank heartily to Prof. Y. Kotake, for his advice and revision also heartily thank to Prof. K. Kodama, for his revision.

REFERENCES

(*1*) Kotake, Y., Jr., and Inada, T., *J. Biochem.*, **40**, 287 (1953)
(*2*) Kotake, Y., and Shirai, Y., *Z. physiol. Chem.*, **295**, 160 (1953)
(*3*) Borchers, R., Berg, C. P., and Whitmann, N. E., *J. Biol. Chem.*, **145**, 657 (1942)
(*4*) Glazer, H. S., Mueller, J. F., Thompson, C., Hawkins, V. R., and Vilter, R. W., *Arch. Biochem. Biophys.*, **33**, 243 (1951)
(*5*) King, E. J., and Garner, R. J., *J. Clini. Pathol.*, **1**, 30 (1947)
(*6*) Kotake, Y. Jr., Inada, T., and Matsumura, Y., *J. Biochem.*, **41**, 255 (1954)

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PHOTOCHEMICAL PHOSPHATE TRANSFER IN GREEN LEAVES. I

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(Received for publication, February 27, 1954)

Although a good deal of attention has recently been directed to the significance of phosphate transfer in the mechanism of photosynthesis, (*1*, *2*, *3*, *4*) the actual role played by phosphate still remains obscure. In the experiments reported below we found that in the leaves of spinach an enzymatic phosphate transfer from α- or β-glycero-phosphate to some phosphate acceptor occurs under the influence of light. The enzymatic system concerned was found to be present in the cytoplasmic fraction, while the effect of light is transmitted by virtue of grana contained in chloroplasts. In view of this fact it may not be amiss to conjecture that the phenomenon is connected, if not essentially, at least circumstantially, to the mechanism of photosynthesis.

Effect of Light on the Liberation of Inorganic Phosphate from Glycerophosphate by Leaf Homogenate

Spinach leaves were ground with the Waring blendor in *M*/50 acetate buffer of pH 6.0 and then centrifuged. The supernatant dark green solution contained cytoplasmic fluid and grana particles, of which the former showed an enzymatic activity to split phosphate from α- and β-glycerophosphate. Several ml. each of the green homogenate were placed in two vessels of the Warburg manometer and, after evacuation, the vessels were vigorously shaken, one in the light and the other in the dark. Illumination was furnished by a 200w. lamp placed 30 cm. above the vessel.

A solution of β-glycerophosphate which had been placed in the side arm, was added to the homogenate suspension (end concentration being *M*/500), and at suitable intervals aliquotes of the reaction mixture were taken out and poured into test tubes containing a small amount of 10 per cent trichloracetic acid (the reaction mixture was evacuated after each sampling). The solution was then diluted with cold water, filtered through ashless filter paper, and the amount of inorganic phosphoric acid was measured by the method of Feigl (*5*) using ammonium molybdate, benzidine and sodium acetate. All procedures for determining phosphate were carried out at a temperature below 5°C. This was necessary to prevent the decomposition of labile

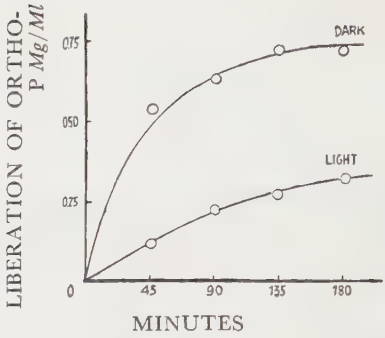

FIG. 1. Effect of light on the liberation of inorganic phosphate from β-glycerophosphate caused by spinach leaf homogenate.

End concentration of glycerophosphate, *M*/500. pH 6.0. Temperature 24°.

phosphate-ester formed by the effect of illumination.

Experiments showed that the liberation of inorganic phosphate from β-glycerophosphate (under anaerobic condition) was remarkably suppressed by the effect of illumination. In Fig. 1 is reproduced a typical example of the experimental results.

Among the several other organic phosphates tested, only α-glycerophosphate was found to show a phenomenon similar to that described above. It was found that ATP, acetylphosphate, and inorganic pyrophosphate were hydrolyzed enzymatically by the leaf homogenate without, however, being influenced by the effect of light.

Effect of Various Factors

Apparent suppression of phosphate liberation from β-glycerophosphate by the effect of illumination occured most distinctly at pH 6.0 and at 24°. The phenomenon appeared to be halted by the effect of 2,4-dinitrophenol, but only in its relatively high concentration such as *M*/500. No effect was observed when DPN or TPN was added to the reaction mixture.

Formation of A Labile Phosphate Ester by the Effect of Illumination.

The data given in Fig. 1 admit of being interpreted in two ways: either (a) the phosphatase activity itself was directly inhibited by light, or (b) the light caused a transfer of phosphate from glycerophosphate to some other substance to form a new organic phosphoric ester. Experiments showed that the latter was the case, and the formation of a new phosphoric ester in the illuminated reaction mixture was shown by the technique of paperchromatogram. The substance which was entirely

* P^{32}-labelled β-glycerophosphate was synthesized according to Bailley (*6*).

absent in the unilluminated reaction mixture showed an *Rf* value that was different from either that of orthophosphate or that of glycerophosphate. Exact value of *Rf* of this new substance could, however, not be determined because of the remarkable lability of the substance. Decisive evidence for the transfer of phosphate from glycerophosphate to the unknown substance was also obtained by using P^{32}-labelled β-glycerophosphate* as the substrate.

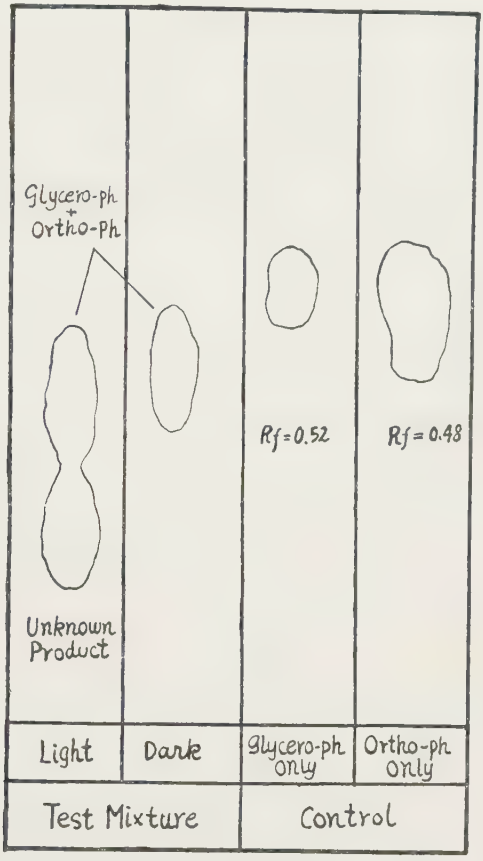

FIG. 2. Paperchromatogram of the unknown substance in admixture with ortho-phosphate and β-glycerophosphate.
Developing solvent: butanol-acetic acid-water.

The reaction mixture, which was ice-cooled after the reaction was completed, was subjected to acid hydrolysis of short duration. As may be seen from Table I,

most of the product was hydrolyzed within 10 minutes, while the β-glycerophosphate was not decomposed at all by the treatment.

TABLE I

Hydrolysis of the Illuminated Reaction Mixture

Each value shows the amount of inorganic phosphate contained, before and after the hydrolysis lasting 10 minutes. Experiment No. 5 shows the values for β-glycerophosphate.

Exptl. No.	Before hydrolysis	After hydrolysis	Whole P
	mg./ml.	*mg./ml.*	*mg./ml.*
1	0.055	0.255	0.35
2	0.15	0.28	0.30
3	0.16	0.23	0.26
4	0.13	0.58	0.42
5	0.065	0.070	—

Significance of Grana in the Reaction Mechanism

Green homogenate of spinach leaves was separated by centrifugation into cytoplasmic fluid and grana particles. The latter was washed several times with the buffer solution. It was found that the phosphatase activity was present in cytoplasmic fluid but not in grana particles. The data given in Table II shows that the liberation of phosphate from glycerophosphate by cytoplasmic fluid was not affected by light in the absence of grana, while a considerable light effect was observed in the reaction mixture in which grana was reunited with the cytoplasmic fluid. It may, therefore, be concluded that the grana plays an essential role in the photochemical process in question.

TABLE II

Significance of Grana in causing the Photochemical Transfer of Phosphate

	Control		Without *Grana*		*Grana* reunited	
	Light	Dark	Light	Dark	Light	Dark
Ortho-Ph. liberated in 180 min. *mg./ml.*	3.25	4.5	2.55	2.8	2.5	4.5

Attempt at Purification of the Enzyme System

Purification of the enzyme system concerned in the process described above was

attempted by the following procedure. The homogenate of spinach leaves prepared at temperatures below 5° was filtered through a cloth, and the filtrate was adjusted to pH 4.5–4.6 by gradually adding small amounts of *M*/10 acetic acid. The precipitates formed, together with the grana, were centrifuged off, and the supernatant was adjusted to pH 6.0 by adding *M*/10 KOH. To this solution ammonium sulphate was added up to 50 per cent saturation and the resulting precipitates were collected by centrifugation and dissolved in a small amount of *M*/50 acetate buffer or pH 6.0, and then, ammonium sulphate was added at 35 per cent saturation and the precipitate was discarded. With this supernatant, the fractionation with ammonium sulphate at 50 per cent saturation was repeated several times, insoluble protein being discarded each time by centrifugation. Finally, the enzyme was dissolved in a small amount of acetate buffer and dialyzed against the same buffer solution in an ice box for 20 hours.

The phosphatase activity of the preparation thus obtained was found to be fairly low. Contrary to our expectation, addition of histidine or divalent metal ions such as Mg, Mn, Ca, Cu, and Fe proved to be ineffective in enhancing the activity. (Ascorbic acid was found to be inhibitive at the concentration of *M*/500). However, a considerable recovery of the phosphatase activity was observed when a deproteinized supernatant of the homogenate was added to the preparation, indicating that some activator for the enzyme is contained in the homogenate. (Fig. 3)

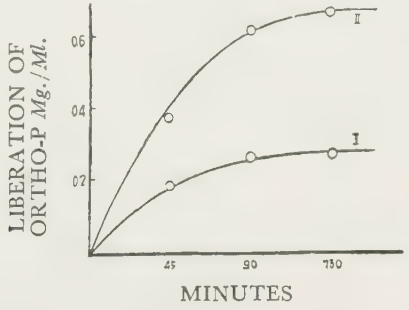

FIG. 3. Activation of phosphatase activity of purified cytoplasmic fluid by the addition of deproteinized supernatant of homogenate. Curve I: control; Curve II: supernatant added.

The enzyme preparation itself showed, even when mixed with *Grana*, no transphosphatase activity on illumination. The latter phenomenon, however, was definitely manifest when the deproteinized supernatant of the homogenate was added to the mixture of enzyme preparation and grana, indicating that the homogenate contains, besides the activator mentioned above, the acceptor of phosphate for the light-induced transphosphorylation. (Fig. 4)

Attempt at Purification of the Phosphate Acceptor

To date, neither the nature of the activator mentioned above nor that of the

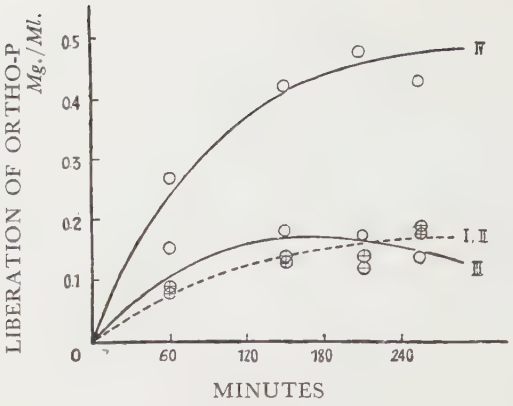

FIG. 4. The effect of the deproteinized supernatant of homogenate on the photochemical transphosphorylation.

Curves I and II: control mixtures in the light and dark; Curves III and IV: test mixtures with added supernatant in the light and dark.

phosphate acceptor has been discerned. By the following procedure, however, we were able to separate the acceptor from the activator and to investigate its nature to some extent.

Leaf homogenate mode with distilled water was heated at about 60° for 10 minutes, and then filtered. On adding solid lead subacetate to the filtrate, yellowish precipitates were formed, which were separated by centrifugation. The precipitates were suspended in a small amount of cold water and decomposed with hydrogen sulfide, which was subsequently driven off by aeration. Before the aeration, a very small amount of octyl alcohol was added to the reaction mixture to prevent vigorous foaming. The supernatant solution was gradually neutralized with *M*/10 NaOH to pH 6.0. The solution obtained, which may be referred to as *Extract 1*, was dark brown in color and contained an appreceable amount of the phosphate acceptor, together with some quantity of the phosphatase activator as is evident from the results shown in Fig. 5.

To *Extract 1* was added $AgNO_3$, and the precipitate formed was suspended in water. To this suspension, saturated NaCl solution was added and the precipitate of AgCl was discarded. The supernatant, which may be called *Extract 2*, contained the phosphate acceptor, but almost no activator, as may be seen from the results given in Fig. 6. *Extract 2* contains still a certain amount of inorganic phosphate which was precipitated by $CaCl_2$. The acceptor which was present in the supernatant solution was precipitated by adding an equal volume of acetone to the solution, and the precipitate was then dissolved in water. Further purification of the acceptor was achieved by the adsorption on characoal, followed by elution with 10 per cent aqueous pyridine and by subsequent removal of pyridine with chloroform

using a separation funnel. The solution of the acceptor thus obtained was slightly yellow at the acidic pH and dark brown at the pH above 6.0. It gave no color reaction either with iron sesquichloride or Millon's reagent. A considerable amount of phosphorous was found in the ash of the sample.

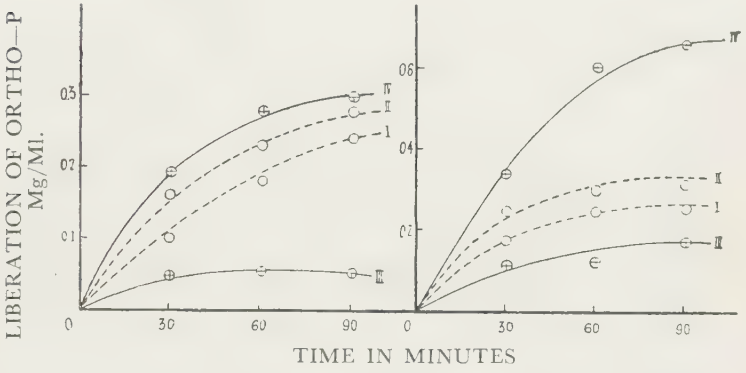

FIG. 5. The effect of *Extract 1* on photochemical transphosphorylation. Curves I and II: control mixtures in the light and dark; Curves III and IV: test mixtures with added *Extract 1* in the light and dark.

FIG. 6. The effect of purified acceptor on photochemical transphosphorylation. Curves I and II: control mixtures in the light and in dark; Curves III and IV: test mixtures with added purified acceptor in the light and dark.

It should be added that the following substances proved ineffective to act as the phosphate acceptor: various sugars, fatty acids, amino acids, AMP, pyruvic acid, oxalacetic acid and yeast extract.

SUMMARY AND DISCUSSION

1. It was found that, when glycerophosphate (α or β) was added to the homogenate of spinach leaves, inorganic phosphate was liberated in larger amounts in the dark than in the light.

2. It was found that in the light the phosphate of glycerophosphate was transfered to some organic acceptor with the formation of some unstable phosphate ester. The process occurring in the light and in the dark may be represented by the following formulae:

In the light: $\text{Gly-Ph} + \text{A} \longrightarrow \text{Gly} + \text{P-A}$

In the dark $\text{Gly-Ph} + \text{H}_2\text{O} \longrightarrow \text{Gly} + \text{P-OH}$

where Gly-Ph is glycerophosphate and A the phosphate acceptor.

3. By separating the leaf homogenate into two fractions—namely, grana and cytoplasmic fluid, it was demonstrated that the phosphatase system is present in the cytoplasmic fluid, while the light effect causing the transphosphorylation is transmitted by virtue of *Grana*.

4. The activity of the glycerophosphatase is enhanced by some unknown organic activator present in the leaf homogenate, which can not be replaced either by histidine or by various divalent metal ions.

5. Attempts were made to isolate the enzyme system, activator and the phosphate acceptor from the leaf homogenate. The acceptor freed from the enzyme, activator and inorganic phosphate could be obtained, but its chemical nature remained unelucidated.

This work was carried out as a part of the program directed by Professor H. Tamiya, and it is our great pleasure to express our gratitude for his kind guidance. A part of the expense of this work was defrayed out of grants from the Ministry of Education and from the Rockfeller Foundation. To these bodies we extend our grateful thanks.

REFERENCES

(*1*) Calvin, M., and Benson, A. A., *Science*, **107**, 467 **108**, 304 (1948)
(*2*) Ochoa, S., and Vishniac, W., *J. Biol. Chem.*, **198**, 501 (1952)
(*3*) Vishniac, W., and Ochoa, S., *Nature*, **167**, 768, (1951)
(*4*) Ochoa, S., and Vishniac, W., *Science*, **115**, 297 (1951)
(*5*) Feigl, I. F., Qualitative Analyse, Tüpel Reaktionen, Akademische Vergessellschaft M. B. H. Leipzig, (1938)
(*6*) Bailly, O., *Ann. de Chem.*, (9), **6**, 96 (1916)

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THE CHOLESTEROL CONTENT OF BLOOD AND TISSUES IN ALLOXAN DIABETES

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Two theories have tentatively been presented on the cause of hypercholestorolemia or fatty liver in alloxan diabetes animals; one attributes to the hypofunction of liver (Stetten and Boxer (*1*)), while the other remarks the importance of insufficient activity of Langerhans' islets (Goldner and Gomori (*2*), and Bailey (*3*)). On the other hand, it is generally accepted that the adrenal glands play an important role in the cholesterol metabolism, and in the case of alloxan diabetes, cretain anatomical changes have already been observed in those glands (Kendall, *et al.* (*4*), and Deane, Shaw and Greep (*5*)).

It would be of interest, with regard to the cause of this hypercholesterolemia, to study the relation between alloxan diabetes and steroid hormones; and accordingly the blood and tissue cholesterol contents were estimated at various stages of the blood sugar levels, using the rat.

METHOD

The experimental and control animals, weighing between 150 and 200 g. were fed on the same diet. The experimental rats were injected intravenously with 50 to 150 mg. of alloxan per kg. of body weight and were killed at various sugar levels. A known amount of organ was dissected out and extracted with 100 times of 95 per cent alcohol. After standing 24 hours at room temperature, it was filtered; 2/3 of the filtrate was used for the free cholesterol determination and the remaing 1/3 was subjected to saponification. Either from the original or from the saponified sample, cholesterol was precipitated as its digitonide. In the former case the phospholipid was precipitated from the digitonide according to the method of Bloor (*6*), and further procedure was performed following Kelsey's method (*7*). The blood for sugar determination from the tail and that for cholesterol estimation were taken from the heart.

TABLE I

The Cholesterol Concentration of Blood and Liver in Control and Alloxan-treated Rats

	Blood sugar (*mg./dl.*)	Blood cholesterol (*mg./dl.*)				Liver cholesterol (*mg./dg.*)			
		Total	Free	Esterified	Ester / Free	Total	Free	Esterified	Ester / Free
Control	129	80.0	44.2	35.8	0.81	150.8	84.2	66.6	0.79
3-hours after alloxan-treatment	158	110.6	33.2	77.4	2.33	166.6	58.6	108.0	1.84
5-hours after alloxan-treatment	31	90.6	34.4	56.2	1.63	189.3	66.6	112.7	1.69
5-days after alloxan-treatment	420	108.8	64.1	44.7	0.70	183.5	123.0	60.5	0.49

It will be seen from the above table that the total cholesterol of blood or liver is, at all stages of alloxan diabetes, significantly higher than that of the control. The ratio of ester and free cholesterol in the blood rises remarkably at the initial hyperglycemic and following hypoglycemic stages, but returns to the control value at the final hyperglycemic period. The similar tendency is seen with the liver, *i. e.* a parallel change of the ratio is observed in the blood and liver.

The Cholesterol Content of Various Tissues at the Final Hyperglycemia of Alloxan Diabetes

So far as the present author is aware, the effect of alloxan on the cholesterol content of the various tissues has not yet been reported and accordingly some measurements of this were porformed with the heart, lung, spleen, kidney and adrenals at the final hyperglycemic stage, 5 days after the alloxan injection.

TABLE II

The Values of Tissue Cholesterol in Control and Alloxan-treated Hyperglycemic Rats

(5-days after alloxan-treatment)

	Control (*mg./dl.*)				Alloxan-rats (*ml./dl.*)			
	Total	Free	Esterified	Ester / Free	Total	Free	Esterified	Ester / Free
Heart	106.4	92.4	14.0	0.15	112.8	112.8	0	0
Lung	126.0	103.5	22.5	0.22	125.5	100.9	24.6	0.24
Spleen	182.6	145.5	27.1	0.19	161.0	130.9	30.3	0.23
Kidney	140.9	99.4	41.5	0.42	134.8	96.2	38.6	0.40
Adrenal	2790.3	1038.3	1752.0	1.67	945.0	495.2	449.8	0.91

Table II shows clearly that alloxan exerts no notable effect on the content of either free or bound cholesterol in the lung, spleen and kidney, while in the heart only the ester portion diminishes and the free cholesterol increases. The adrenal grands, which contain normally an enormous amount of cholesterol and the major part of it belongs to the ester form, show after the alloxan treatment a remarkable decrease of total cholesterol and especially of the esterified from, indicating very probably a considerable removal of this substance from the adrenals. Hence one may assume that at any stage of blood sugar level, not particularly at the final hyperglycemic phase, a large loss of cholesterol would have taken place, if tested.

The data given in Table III show that the loss of cholesterol, especially of its

Changes of the Cholesterol Content of Adrenal at Various Blood Sugar Levels after Alloxan Injection

TABLE III

The Values of Cholesterol in Adrenals of Control and Alloxan-Treated Rats at Various Blood Sugar Levels

		Control	After 5-hours	After 2-days	After 5-days
Blood sugar	*mg./dl.*	177	311	150	455
Total-Cholesterol	*mg./dl.*	2356.6	1323.0	1618.5	1139.2
Free-Cholesterol	*mg./dl.*	726.5	394.0	569.3	437.1
Ester-Cholesterol	*mg./dl.*	1630.1	929.0	1049.2	702.1

ester form, from the adrenals taken place soon after the alloxan administration.

DISCUSSION

It is interesting to note the fact that a large loss occured in adrenals of animals in the initial hyperglycemic period of alloxan diabetes and that the ester form is leargely lost selectively rather than the free form.

Various explanations have been presented to elucidate the initial hyperglycemia of the alloxan diabetes animals: thus Duff (*8*), Ishii (*9*), Nagahama (*10*), Uoji (*11*), *etc.* assumed its origin to the adrenal medulla, while Kirschbaum, Wells and Molander (*12*), Kosaka (*13*), *etc.* attributed it to a hyperactivity of the adrenal cortex function; on the other hand, Houssay and his associates (*14*), Kosaka (*13*), *etc.* insisted that the anterior pituitary plays an important role, and yet others, like Okamoto (*15*) considered the diminished insulin effect (as being due to destruction of the β cells of islets: or again the stimulation of the α cells of islets was also postulated (Thorogood and Zimmermann (*16*)).

The present finding that during the intial hyperglycemic stage in alloxan diabetes rats cholesterol, the precursor of the steroid hormones, diminishes appreciably in the adrenals, may indicate an intimate relation existing between this hyperglycemia and an abnormal activity of the pituitary-cortical hormones.

SUMMARY

The cholesterol content of the blood and the various tissues of alloxan treated rats were estimated at different stages of blood sugar levels.

1. In blood and liver, the total cholesterol increased through all stages of blood sugar levels, whereas the higher ester contents was observed at the initial hyperglycemic and the secondary hypoglycemic stage.

2. The total cholesterol in lung, kidney, heart and spleen showed no significant change. In the heart cholesterol ester decreased, while free cholesterol increased. Such change of ratio was, however, not observed in other organs.

3. Treatment with alloxan reduces rapidly and remarkably the total cholesterol, especially the esterified, in the adrenal glands.

4. In the stage corresponding to the initial hyperglycemic stage of alloxan rat, a large loss of total cholesterol, especially of the ester form, was found in the adrenals. This fact seems to indicate that there exists an intimate relationship between initial hyperglycemia and an abnormal activity of pituitary-cortical hormones in the case of alloxan rat.

REFERENCES

(*1*) Stetten, D., Jr., and Boxer, G. E., *J. Biol. Chem.*, **156**, 271 (1944)
(*2*) Goldner, M. G., and Gomori, G., *Endocrinol.*, **33**, 297
(*3*) Bailey, C. C., and Bailey, O. T., *J. Am. Med. Assoc.*, **122**, 1165 (1943)
(*4*) Kendall, F. E., Meyer W., Lewis, L., and Victor, J., *Proc. Soc. Exptl. Biol. Med.*, **60**, 190 (1945)
(*5*) Deane, H. W., Shaw, J. H., and R. O. Greep, *Endocrinol.*, **43**, 133 (1948)
(*6*) Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929)
(*7*) Kelsey, F. E., *J. Biol. Chem.*, **127**, 15 (1939)
(*8*) Duff, G. L., *Am. J. Med. Sci.*, **210**, 381 (1945)
(*9*) Ishii, T., *J. Biochem.*, **38**, 37 (1951)
(*10*) Nagahama, S., *Folia Endocrinol. Japan*, **28**, 279 (1952)
(*11*) Uoji, S. *Folia Pharmacol. Japan*, **46**, 86 (1950)
(*12*) Kirschbaum, A., Wells, J. T., and Molander D. *Proc. Soc. Exptl. Biol. Med.*, **58**, 267 (1945)
(*13*) Kosaka, T., *Tokyo Ijishinshi* (in Japanese), **59**, 267 (1952)
(*14*) Houssay, B. A., *Cand. Med. Assoc.*, **56**, 519 (1947)

(*15*) Okamoto, K., *Acta Schol. Med. Univ. Kioto*, **27**, 43 (1949)
(*16*) Thorogood, E., and Zimmerman, B., *Endocrinol.*, **37**, 191 (1945)

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DETECTION OF NUCLEIC ACID COMPONENTS ON PAPER CHROMATOGRAM

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(Received for publication, March 11, 1954)

Since Vischer and Chargaff (*1*) applied the paper partition chromatography to the separation and quantitative analysis of purine and pyrimidine bases of nucleic acids, this technique was frequently used to the identification and quantitative analysis of nucleic acid components. They petected the spots of purine and pyrimidine bases on the chromatogram by fixing them with mercury salts and converting the latter to the sulfide. But this procedure is very troublesome.

Since Holiday and Johnson (*2*), Markham and Smith (*3*), and Carter (*4*) reported a method to detect purines, pyrimidines, and nucleosides, and nucleotides on the chromatogram by using the ultraviolet irradiation, this method has been generally employed.

The authors also tried to detect the spots of nucleic acid components by irradiating them with the ultraviolet light filtered through the aqueous solution saturated with nickel and cobalt sulfate following to the recipe reported by Markham and Smith (*3*). Markham and Smith used a quartz mercury vapour lamp of 200 v., d. c., 450 w. as the source of ultraviolet light, and then this light was filtered through two quartz filters containing dry chlorine and a aqueous solution of nickel and cobalt sulfate. But in their procedure, the spots on chromatogram were said to be invisible to the naked eyes, so they detected them by taking photograph. But in our procedure, the spots of nucleic acid components were made visible to the naked eyes by using an adequate light source, and our apparatus (detector) may be made also in Japan very easily and cheaply.

APPARATUS

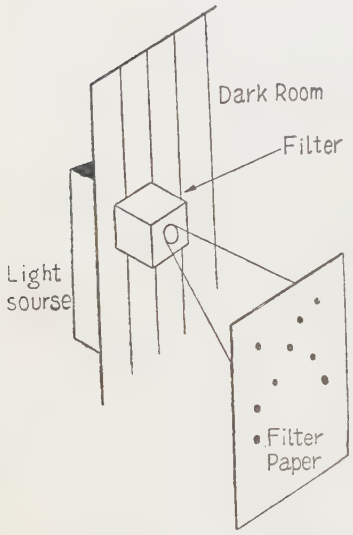
Source of Ultraviolet Light—As the light source, the authors used a low voltage mercury lamp manufactured by Tokyo-Shibaura Denki Company (Matsuda's sterilization lamp GL-15, 100 v., a. c., 15 w.). The voltage involved here is lower than that of the

apparatus used by Markham and Smith (*3*).

Filter—The salt solution described by Markham and Smith (*3*) was used for the filter. It was prepared by dissolving 350 g. of nickel sulfate and 100 g. of cobalt sulfate in 1 litre of water and brought into a glass vessel having a thickness of 30 mm., two windows of which were made out of quartz.

The light source was settled on the outside of a wooden wall of the dark-room and the light was introduced into the filter set attached to the inside of the wall of the dark-room as illustrated in Fig. 1.

EXPERIMENT



Paper chromatography was performed in the cylindlical glass vessel (47 cm. high and 22 cm. diameter). Filter paper used was Toyo Roshi No. 50 (40×40 cm.).

The following solvent systems were employed for the development of the paper chromatograms: (a) butanol saturated with water, (b) butanol, acetic acid, and water (ratio of 4 : 1 : 1), (c) butanol saturated with 10 per cent aqueous solution of urea, (d) two-phase solvent system described by Carter, *i. e.*, 5 per cent monobasic potassium phosphate, 5 per cent sodium phosphate, or 5 per cent sodium citrate adjusted to desired pH with ammonium hydroxide, and isoamyl alcohol was overlapped on this salt solution.

The development with the butanol solvent system was carried out according to the usual techniques of paper chromatography. When two-phase solvent system was used, the development was carried out as described by Carter (*4*).

After the development finished, the filter paper was dried and exposed to the ultraviolet detector. The spots of purines, pyrimidines and their derivatives were shown on chromatogram as dark paches against the background of paper fluorescence. In accordance with the description of Wieland and Bauer (*5*), the spots were more sensitively observed by spraying on the paper 0.005 per cent fluorescein dissolved in 0.5 *N* ammonia. By means of this procedure, the spots were turned to the visible in amount over 1 γ/cm^2. When the solution of fluorescein did not be sprayed, the lower limit of detection was 5 γ/cm^2. of each components.

The compounds used in this study, adenine, xanthine, hypoxanthine, adenosine, uridine, adenosine-5′-phosphate, sodium guanylate, and cytidylic acid were purchased from Schwarz Laboratories, and the others were synthesized or prepared in our laboratory.

Spectrophotometric Examination of Light Source and Filter

Light Source—Matsuda's sterilization lamp shows a specific type of emission spectrum, particularly the emission at 2537 Å, 3132 Å, and 2654 Å are very intense.

Absorption Spectra of Filter Solution—

(a) Nickel sulfate solution ($NiSO_4 \cdot 7H_2O$ 350g./*l.*, d=3.0 cm.). The absorption spectrum of this solution is shown in Fig. 2, Curve a. The region from 222 to 338 mμ is transmitted, and waves from 338 to 470 mμ and shorter waves than 222 mμ are absorbed.

(b) Cobalt sulfate solution ($CoSO_4 \cdot 7H_2O$ 100 g./*l.*, d=3.0 cm.). Its absorption spectrum is shown in Fig. 2, Curve b. From 235 to 440 mμ are transmitted and the visible rays are absorbed.

(c) The mixture of nickel and cobalt sulfate have the absorption spectrum as shown in Fig. 3 (Curve a), *i.e.*, from 235 to 335 mμ are transmitted and other regions

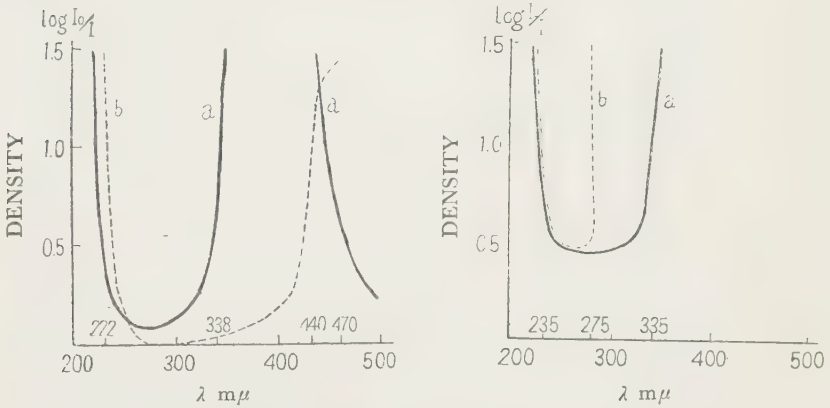


Fig. 2. Absorption spectra of nickel sulfate and cobalt sulfate.
Curve a: $NiSO_4 \cdot 7H_2O$, C=350 g./*l.* d=3.0 cm.
Curve b: $CoSO_4 \cdot 7H_2O$, C=100 g./*l.* d=3.0 cm.

Fig. 3. Absorption spectra of filter. Curve a: Aqueous solution of nickel and cobalt sulfate, Curve b: Chlorine.

are absorbed. Curve b of Fig. 3 shows the absorption spectrum of chlorine. If chlorine be used in addition to the above salt solution, as described by Markham and Smith, the excellent filter can be obtained, which transmits only waves at 235–275 mμ. But the spots on paper chromatogram are well enough visible without using chlorine.

RESULTS AND DISCUSSION

The Rf values obtained are given in Table I.

TABLE I

Rf-values of Nucleic Acid Components

	Butanol-water, pH 7.0	Butanol-acetic acid-water (4 : 1 : 1), pH 3.0	Butanol-urea, pH 6.8	5% KH_2PO_4 isoamyl alcohol, pH 6.8	5% Na_2HPO_4 isoamyl alcohol		5% Na citrate isoamyl alcohol	
					pH 7.2	pH 8.0	pH 6.8	pH 8.0
Adenine	0.44	0.53	0.42	0.41	0.40	0.39	0.37	0.37
Guanine	0	0						
Xanthine	0.18	0.26						
Hypoxanthine	0.32	0.36	0.29	0.55	0.56	0.56	0.56	0.52
Uracil	0.40	0.43	0.35	0.71	0.72	0.68	0.75	0.68
Cytosine	0.24	0.34	0.23	0.73	0.72	0.68	0.75	0.67
Thymine	0.58	0.59	0.52	0.72	0.72	0.68	0.75	0.68
Adenosine	0.29	0.40	0.26	0.53	0.51	0.51	0.54	0.49
Inosine	0.13	0.18	0.10	0.61	0.61	0.60	0.61	0.57
Guanosine	0.17	0.22	0.13	0.71	0.71	0.68	0.74	0.72
Uridine	0.23	0.29	0.20	0.79	0.78	0.80	0.83	0.80
Adenosine-5-phosphate	0	0.027	0	0.69	0.76	0.73	0.75	0.73
Na guanylate	0.10	0.18	0	0.82	0.77	0.77	0.77	0.72
Cytidylic acid	0	0	0	0.86	0.88	0.86	0.89	0.71

In general, the butanol solvent system is adequate for chromatographic separation of purine and pyrimidine bases, and nucleosides, but two-phase solvent system affords convenience to distribute the nucleotides on chromatogram. Rf values used to very with the experimental conditions such as temperature and thickness of solvent layers and so on, but the relation of spots to each other remains unaltered. When two-phase solvent system is employed for the development, the spots on chromatogram have a tendency to diffuse, and they can not be detected in less than 10 γ/cm^2.

As all the nucleic acid components (nucleotides, nucleosides, and bases) have ultraviolet absorption in the range from about 250 to 280 mμ, so the suitable light source for their detection on paper chromatogram must have emission spectra at this range, and the visible region must be as weak as possible. For this purpose, a low voltage mercury resonance lamp such as Matsuda's sterilization lamp, is most suitable. When a higher voltage mercury lamp was used as the light source, the spots on chromatograms could not be detected with the naked eye. As the condition of an ideal filter, it must transmit only rays from nearly 250 to 280 mμ, and moreover absorb other waves than these. So the filter such as is prescribed by Markham and Smith (3) is satisfactory for this purpose.

SUMMARY

The spots of nucleic acid components on paper chromatogram were detected by an ultraviolet detector. This detector consists of a low voltage mercury lamp (Matsuda's sterilization lamp GL-15, 100 v., 15 w.) as the light source and a solution of nickel and cobalt sulfate described by Markham and Smith as the filter. In this paper, the apparatus, the spectrophotometric research in the detector and Rf values of nucleic acid components were described.

The lower limit of detection with this detector was 1 to 5 γ of each compound.

This work was aided by a grant from the Scientific Research Fund of the Ministry of Education.

REFERENCES

(*1*) Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176**, 703 (1948)
(*2*) Holiday, E. R., and Johnson, E. A., *Nature*, **163**, 216 (1949)

(3) Markham, R., and Smith, J. D., *Nature*, **163**, 250 (1949)
(4) Carter, C. E., *J. Am. Chem. Soc.*, **72**, 1666 (1950)
(5) Wieland, J., and Bauer, L., *Angew. Chem.*, **63**, 512 (1951)

The Journal of Biochemistry, Vol. 41, No. 4, 1954

BIOCHEMICAL STUDIES ON INTERMEDIATE METABOLISM OF VITAMINS

I. ON INTERACTION OF VITAMIN B_1 AND SULFHYDRYL GROUP IN PROTEIN MOLECULES.

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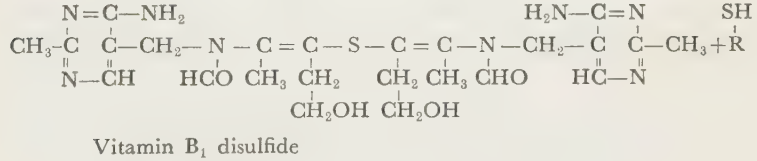
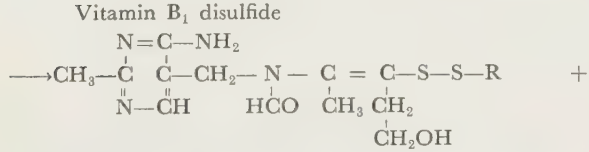
(Received for publication, March 7, 1954)

In 1941, it was found by O. Zima that vitamin B_1 disulfide was converted to vitamin B_1 by the action of cysteine or reduced glutathione, and the sulfhydryl group also may be calculated by treating with vitamin B_1 disulfide and estimating the micro-amount (0.2–0.5 γ) of vitamin B_1 in the obtained solution with thiochrome method. In 1950, Sahashi reported that this principle may be also applied for micro-determination of sulfhydryl group in protein molecules such as activated papain or egg albumin denatured by urea. Afterwards, in 1952, Matsukawa carried out further studies on Zima's reaction of cysteine and vitamin B_1 disulfide, and found a new intermediate dithio-compound B_1-SS-cysteine by paper chromatography.

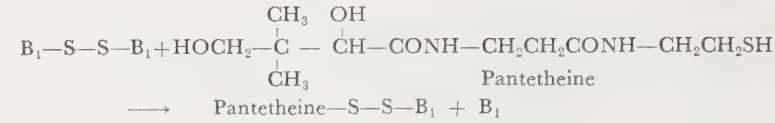
Guided by these facts, the authors have recently attempted biochemical studies on vitamin B_1 in intermediate metabolism and at first, interaction of vitamin B_1 disulfide and activated sulfhydryl group in peptides was tested by paper chromatography.



Vitamin B_1 disulfide



B_1—S—S—R R=peptides, proteins or related compounds.



Intraction of Glutathione and Vitamin B_1 disulfide

At first, the paper chromatogram of the following S-containing compounds was studied by using various colouring reactions. The R_F-values were standardized as shown in Table I.

TABLE I

Paper Chromatogram of Some S-containing Compounds

Sample	R_F-value	With K iodo-platinate§	With thio-chrom	With Na-nitro-prusside	With ninhydrin
Vitamin B_1	0.42	blue	+		
B_1-disulfide	0.44	light reddish purple	—	orange*	
Glutathione (SH)	0.16	white	—	red	purple
Glutathione (S-S)	0.04	yellow	—	orange*	purple
Cysteine	0.24	white	—	red	purple
Cystine	0.05	yellow	—	orange*	purple

Solvent: BuOH : AcOH : H_2O = 4 : 1 : 1

§ Back ground became pink.

* Treated with NaCN.

M/100 glutathione (SH) was dissolved in *N*/10 phosphate solution (pH 7.4–7.5) and *M*/100 vitamin B_1 disulfide was added at 37°. After 10 minutes the reaction mixture thus obtained was tested by paper chromatography. The results obtained are given in Table II.

At the end of the experiment, the spots (R_F-value 0.46, 0.15) were treated with cysteine solution and the recovery of vitamin B_1 was recognized respectively. From these facts, the authors have supposed to have prepared a new dithio-compound, B_1-S-S-glutathione. Further confirmation will be reported before long.

Interaction of SH-activated Protein and Vitamin B_1

Several experiments with Merck's papain, egg-albumin and serum-albumin were

TABLE II

Paper Chromatogram of B_1-S-S-glutathione System

	R_F-value	With thiochrome	With nitroprusside	With K iodoplatinate
Glutathione (S-S)	0.04	−	+	+
Vitamine B_1	0.44	+	−	+
B_1-S-S-B_1	0.46	−	+	+
B_1-S-S-glutathione	0.15	−	+	+

Solvent: BuOH: AcOH: H_2O=4: 1: 1

carried out. Sulfhydryl group in papain was carefully activated with HCN, and egg-albumin or serum-albumin with guanidine.

To 1 ml. of 2 per cent water solution of papain was added 0.5 ml. of 3 per cent HCN, and the solution was adjusted to pH 5.5-6.2 at 37°C. After 1.5 hours, the above solution was treated with vitamin B_1 disulfide for 15 minutes. Egg-albumin or serum-albumin was activated by 5 *M* guanidin solution at pH 4.0-4.5 at 37°. After 1.5 hours, the solution was treated with vitamin B_1 disulfide as mentioned above for 20 minutes, and the reaction products were tested by paper chromatography. After drying each portion was extracted with *M*/10 Na salicylate solution and subsequently treated with cysteine, and vitamin B_1 was determined by thiochrome-bromocyanogen method. The results are shown in Tables III and IV.

TABLE III

Paper Chromatogram of B_1-S-S-papain

	B_1-S-S-papain	B_1	B_1-S-S-B_1
R_F-value	0	0.42	0.44
Reaction product	±	++	−
Reaction product treated with cysteine	+	++	+

Solvent: BuOH: AcOH: H_2O=4: 1

Paper Chromatography of B_1-S-S-pantetheine System

In 1951, Snell reported on the paper chromatography of pantetheine-cysteine system. Recently, the present authors have repeated these experiments and the mixed disulfide was confirmed by ninhydrin reaction to be of the probable formula "pantetheine-S-S-cysteine" (Table V). Then, the authors carried out experiments with vitamin B_1 disulfide and pantetheine freshly prepared.

TABLE IV

Paper Chromatogram of B_1-S-S-albumin

	B_1-S-S-egg albumin B_1-S-S-serum albumin	B_1	B_1-S-S-B_1
R_F-value	0~0.05	0.19	0.22
Reaction product	+	++	−
Reaction product treated with cysteine	++	++	+

Solvent: 50% acetone containing 1.5% NaCl.

Vitamin B_1 disulfide was treated with concentrate of pantetheine at pH 6.5-7.0 at 30°. After 10 minutes the reaction product was tested by paper chromatography as mentioned above. (Table VI)

TABLE V

Chromatogram of Pantetheine-cysteine System

	Cystine	Pantetheine	Pantetheine-S-S-cysteine
R_F-value	0.05	0.42	0.89

Solvent: BuOH: AcOH: H_2O=4:1:1

TABLE VI

Chromatogram of B_1-S-S-pantetheine System

	B_1	B_1-S-S-B_1	B_1-S-S-pantetheine	Pantetheine
R_F-value	0.32	0.45	0.64	0.88

Solvent: BuOH: AcOH: H_2O=4:1:1

SUMMARY

1. Interaction of vitamin B_1 disulfide and activated sulfhydryl groups in peptides or protein molecules was studied and probable forms of B_1-S-S-glutathione, B_1-S-S-papain, B_1-S-S-eggalbumin and B_1-S-S-serum albumin were observed by paper chromatography as shown in Tables I–VI.

2. Interaction of vitamin B_1 disulfide and pantetheine also seems to produce B_1S-S-pantetheine.

From these findings, probable reaction in intermediate metabolism of vitamin B_1 is discussed.

The authors wish to express their sincere thanks to Prof. Y. Kuno (Chairman, Committee on Vitamin B Research). The costs of this research have been defrayed from the Department of Education to which the authors' thanks are due.

REFERENCES

(*1*) Zima, O., Ritsert, K., and Moll. Th., *Z. physiol. Chem.*, **267**, 210 (1941); Matsukawa, T., *Proc. Comm. Res. Vit. B.*, **58**, 7 (1952)

(*2*) Sahashi, Y., and Shibasaki, H., *J. Agr. Chem. Soc. Japan*, **25**, 57 (1951)

(*3*) Gregory, J. D., and Lipmann, F., *J. Am. Chem. Soc.*, **74**, 4017 (1952)

The Journal of Biochemistry, Vol. 41, No. 4, 1954

ON THE KINETICS OF THE HUMAN BLOOD CHOLINESTERASE

IV. THE INHIBITION OF CHOLINESTERASE BY SODIUM SALICYLATE

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(Received for publication, March 23, 1954)

It was shown in previous paper (*1*) that cholinesterase is inhibited by urethane in two qualitatively different ways according to the concentration of the latter applied; namely the inhibition is reversible in lower concentrations, and irreversible in higher concentrations of urethane. The latter reaction was assumed by the author to be due to the denaturation of enzyme protein by urethane.

Present communication deals with the effect of sodium salicylate on human erythrocyte cholinesterase. Also in this case, two different modes of inhibition were found to occur.

Similar phenomena have recently been reported by Goldstein (*2*) with $HgCl_2$ on cholinesterase and also by Johnson *et al.* (*3*) with urethane on invertase. On the other hand, the reaction between hemoglobin and sodium salicylate or sodium benzoate has been reported by Kikuchi (*4*), and Tsushima (*5*), in which they showed that a reversible grade of denaturation (perturbation) seems to occur in the protein part of the hemoglobin molecule. Therefore, it is of most interest to investigate whether the proteins from different sources may be effected by perturbators, as sodium salicylate, sodium benzoate or urethane in a similar manner.

EXPERIMENTALS

The preparation of cholinesterase and the measurement of its activity were carried out by the method previously reported (*6*). Acetylcholine chloride served as the substrate. pH of the reaction medium was held at 7.4 throughout the whole experiments. Temperature was held at 37.5°, except in the experiments of temperature change. Sodium salicylate solution was added to the enzyme solution (side arm) in some

of the experiments and also to the substrate solution (main vessel) in the other. In the former, except in experiments on varied incubation time, the enzyme solution was first incubated with sodium salicylate for 10 minutes at room temperature and then for 10 minutes at 37.5°. The activity was expressed in terms of cmm. CO_2 evolved every 10 minutes. The effect of sodium ion upon cholinesterase activity, observed by Alles and Hawes (*7*), could not be proved in the concentrations of sodium salicylate used in the present experiment.

RESULTS AND DISCUSSIONS

Reversibility of the Salicylate Inhibition

Changes in the activity of cholinesterase were measured every 10 minutes with 0.0025 *M.* acetylcholine solution containing sodium salicylate in varied concentrations. Fig. 1 (II-VIII) shows a plot of enzyme activity thus measured against time. The activity of enzyme decreases gradually with increasing sodium salicylate concentration. The reaction proceeded linearly in each sodium salicylate concentration. Now, enzyme was first incubated (for 10 minutes at room temperature and then for 10 minutes in thermostat) with salicylate of varied concentrations as indicated in Fig. 1 (II–VI) and the substrate solution was then added, so that the final salicylate concentrations were lowered to 1/26 of the initial concentrations. The activity was then found to be restored practically to the level of the control value (Fig. 1, I). But the activity measured after the incubation with salicylate in the concentrations as indicated in Fig. 1. (VII–VIII) were proved to deviate from control value, because of the reason as discussed in the later section. From the results of Fig. 1. (II–VI), it was proved that the inhibition of red cell cholinesterase by sodium salicylate in these concentrations is reversible and that the equilibrium of the inhibition reaction seems to be established in relatively short time, since the reaction proceeded linearly from the starting point.

The Effect of Substrate Concentrations upon the Inhibition by Salicylate

The degree of inhibition, H, may be defined as follows,

$$H = 1 - v_g/v_o \qquad \text{Eq. (1)}$$

where v_o and v_g are the velocity in the absence and presence respectively of sodium salicylate. The relationship between H and the reciprocal of logarithm of sodium salicylate concentrations was obtained from the plot of Fig. 1 in a S-shaped curve as shown in Fig. 2 III (full curve). Now, in the reversible inhibition H can be generally expressed by

$$H = \frac{G^n}{\phi^n + G^n} \qquad \text{Eq. (2)}$$

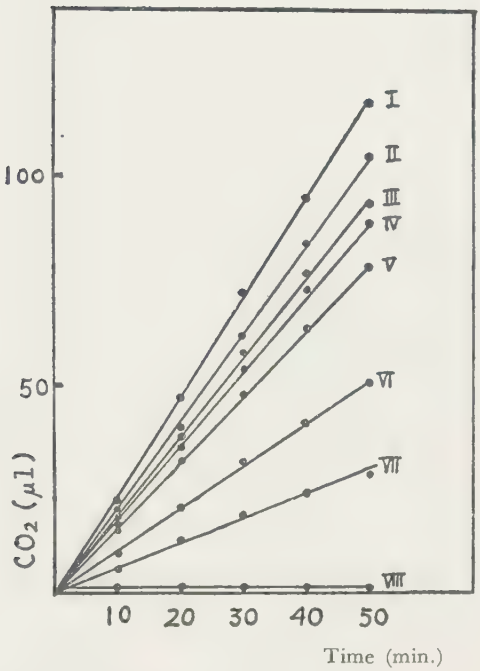

Fig. 1. Inhibition of cholinesterase by sodium salicylate, expressed by activity-time relationship.

Final molarity of sodium salicylate was as follows; I, 0; II, 0.0612; III, 0.102; IV, 0.123; V, 0.153; VI, 0.204; VII, 0.245 and VIII, 0.306.

where G is the inhibitor concentration, ϕ is a constant which corresponds to the inhibitor concentration at 50 per cent inhibition.

As may be seen from Fig. 2, the curves below about 0.5 of H fits to the theoretical curve when n=2 (broken line).

Experimental points above 0.5 of H deviated from the theoretical curve because of the reason as discussed in the later section. Curve I, II and IV indicate a similar relationship between H and -log of salicylate concentration in varied substrate concentrations.

From this results, the inhibition reaction within the fit of Eq. 2, which is a reversible inhibition, can be suggested as that in which two molecules of salicylate combine with one molecule of enzyme. It was further proved that the experimentally obtained values of ϕ within the fit of Eq. 2 in Fig. 2 are a function of the concentration of substrate. The relationship between the values of ϕ and the

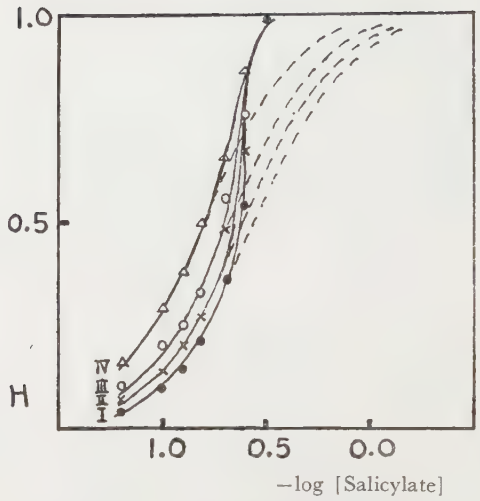

FIG. 2. The fit of the calculated sigmoid curve of second order to the experimental data. Ordinate, the degree of inhibition; abscissa, logarithm of sodium salicylate concentrations. Shift of the curve with variation of the substrate concentrations: I, 0.01 *M*; II, 0.005 *M*; III, 0.0025 *M*; IV, 0.00125 *M*.

TABLE I

The Relationship between the Values of Φ and the Concentrations of Substrate

Substrate concentration	ϕ
mole/liter	*mole/liter*
0.01	0.309
0.005	0.245
0.0025	0.200
0.00125	0.156

concentration of substrate are shown in Table I. The values of ϕ decreases gradually with decreasing substrate concentrations. From the results above mentioned, the reversible inhibition of cholinesterase by sodium salicylate may be represented by the following equations:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad \text{Eq. (3)}$$

$$ES \xrightarrow[k_3]{} E + P \qquad \text{Eq. (4)}$$

$$ES + S \underset{K'm}{\rightleftharpoons} ESS \qquad \text{Eq. (5)}$$

$$E + 2G \underset{Kg}{\rightleftharpoons} EG_2 \qquad \text{Eq. (6)}$$

$$ES + 2G \underset{K'g}{\rightleftharpoons} ESG_2 \qquad \text{Eq. (7)}$$

where E, S, P and G are cholinesterase, acetylcholine, reaction product and sodium salicylate, k_1, k_2, and k_3 being the rate constants, K'm, Kg and K'g are the dissociation constants of Eqs. (5), (6), and (7), respectively.

Based on these representations, the stationary rate of hydrolysis in the presence of salicylate is given by

$$v_g = \frac{K_3[\sum E]}{1 + \frac{Km}{[S]} + \frac{[S]}{K'm} + [G]^2\left(\frac{Km}{Kg[S]} + \frac{1}{K'g}\right)} \qquad \text{Eq. (8)}$$

where $[\sum E]$ is the tatal concentration of the enzyme and Km is the Michaelis constant $(=(k_2+k_3)/k_1)$*. If G=0, the rate of hydrolysis will be

$$v_o = \frac{k_3[\sum E]}{1 + \frac{Km}{[S]} + \frac{[S]}{K'm}} \qquad \text{Eq. (9)}$$

Introducing the values of v_g and v_o in Eq. (1) H can be expressed as follows:

$$H = \frac{[G]^2}{\frac{Kg\ K'g\ (K'm\ [S] + Km\ Km' + [S]^2)}{K'm\ (Km\ K'g + Kg\ [S])} + [G]^2} \qquad \text{Eq. (10)}$$

From the comparison of this equation with Eq. (2), the value Φ has the following implication:

$$\Phi^2 = \frac{1 + \frac{Km}{[S]} + \frac{[S]}{K'm}}{\frac{Km}{Kg[S]} + \frac{1}{K'g}} \qquad \text{Eq. (11)}$$

When one of the reactions Eqs. (6) or (7) is negligible or in case of Kg=K g, ψ can be derived from Eq. (11) as follows:

$$\psi^2 = K'g\ (1 + \frac{Km}{[S]} + \frac{[S]}{K'g} \qquad \text{Eq. (12)}$$

$$\psi^2 = Kg\ (1 + \frac{[S]}{Km} + \frac{[S]^2}{KmK'm} \qquad \text{Eq. (13)}$$

(* The values of Km and K'm were obtained to be 3.7×10^{-4} mole/liter and 1.2×10^{-2} mole/liter respectively in the previous reports (6)).

$$\phi^2=Kg\ (1+\frac{[S]^2}{Km\ K'm+K'm\ [S]} \qquad \text{Eq. (14)}$$

The values of Kg or K'g calculated from these three equations were not obtained as constant.

As may be seen from Table II, the values of Kg and K'g can be obtained as constant (except the value of the last in the third column), only when the values are calculated on the basis of Eq. (11) and from the experimental data on two different substrate concentrations. It is shown in Table III that the observed values of ϕ are in good agreement with the values of ϕ calculated from Eq. (11) by introducing the values of Kg and K'g into the equation.

Table II

The Values of Kg and K'g which are Calculated from Eq. (11) in Various Substrate Concentrations

Substrate concentration	Kg	K'g
mole/lit.	*mole/lit.*	*mole/lit.*
0.01		
0.005	7.06×10^{-3}	7.02×10^{-2}
0.01		
0.0025	$7.75\times$ //	$6.75\times$ //
0.01		
0.00125	$6.81\times$ //	$7.06\times$ //
0.005		
0.0025	$8.15\times$ //	$6.35\times$ //
0.005		
0.00125	$6.70\times$ //	$7.19\times$ //
0.0025		
0.00125	$6.24\times$ //	$9.80\times$ //
Average	7.12×10^{-3}	7.36×10^{-2}

Tabe III

The comparison of Observed Values of ϕ with Calculated Values of That in Various Substrate Concentrations.

Substrate concentration	ϕ obsvd.	ϕ calcd.
mole/lit.	*mole/lit.*	*mole/lit.*
0.01	0.309	0.316
0.005	0.245	0.249
0.0025	0.200	0.199
0.00125	0.156	0.159

These results indicates that the reversible inhibition of cholinesterase by salicylate is non-competitive, in which two molecules of the latter seem to be combined with one molecule of the enzyme, and that the combination of one substrate molecule with enzyme molecule may hinder the binding of salicylate molecules to enzyme molecule. It is suggested that an interaction may be present between the binding groups in the enzyme molecule which combine with substrate on one hand and salicylate on the other.

The Inhibition by Sodium Salicylate in Its Higher Concentrations

All the results described so far were obtained by the determination of remaining activities of enzyme in which enzyme was added to the substrate solution, the latter had been preliminary incubated with sodium salicylate solution. When, however, the enzyme was previously incubated with sodium salicylate, namely 10 minutes at room temperature and further 10 minutes in thermostat, no inhibition has been

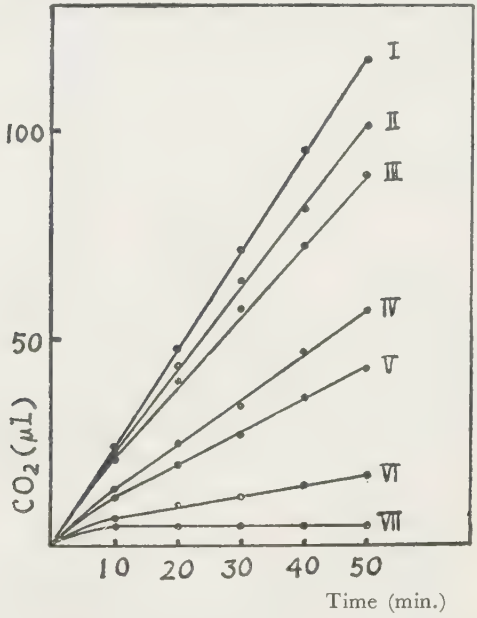

Fig. 3. Relationship between time and the remaining enzyme activity after incubation with sodium salicylate. Molarity of sodium salicylate during the incubation was as follows: I, 0; II, 0.243; III, 0.257; IV, 0.274; V, 0.292; VI, 0.312 and VII, 0.329, final concentrations of sodium salicylate then being lowered to 1/26 of the concentration during incubation.

proved in those concentrations as were applied in Fig. 1 (II-VI). After further increase of salicylate concentration, however, inhibition occured even under the same condition. The remaining activities thus estimated are shown in Fig. 3, in which the final concentrations of salicylate being reduced to 1/26 of those initially incubated. It has been proved in Fig. 1 that salicylate does not act inhibitory when the incubation of enzyme with salicylate was preliminary undertaken with these concentrations. As indicated in Fig. 3 (II-VII), the remaining activity decreased with increase of incubated salicylate concentration and the hydrolysis by remaining activity proceeded linearly only after 10 minutes. In this case a linear relation seems not to be established until 10 minutes. This point makes a distinction from the process inhibited by urethane. In the present stage of investigation, however, it can not be plausibly explained, why the velocity in the first 10 minutes is not in accord with that in the later stage. As the matter of fact, however, the enzyme could not be reactivated by dilution with substrate solution. The results in Fig. 3 indicates that the inactivation by salicylate in its higher level is predominantly an irreversible reaction. The relationship between the degree of inhibition and the reciprocal of logarithm of the salicylate concentrations is shown in Fig. 4 (curve, B); the curve is wholly different from that given in Fig. 2 (Curve, A). This relation is qualitatively the same as that in the inhibition by urethane. It is readily understood that the deviation of the experimental plots in Fig. 2 from the theoretical

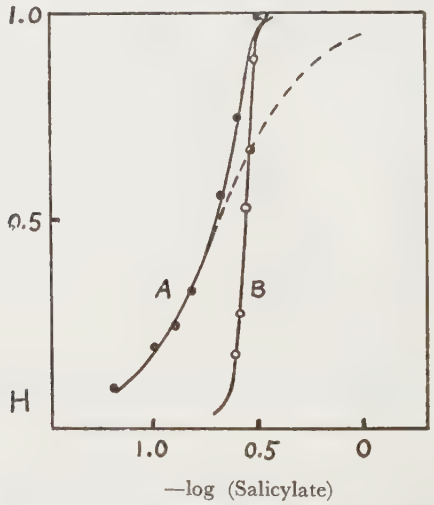

Fig. 4. The degree of inhibition by sodium salicylate in its lower concentrations (A) were compared with those in its higher levels (B). Ordinate, the degree of inhibition; abscissa, logarithm of sodium salicylate concentrations. Curves A and B were obtained from the date given in Fig. 1 and 2, respectively.

curve above H=0.5 is due to the reflection of curve B.

The Effects of Substrate Concentrations against the Inhibition by Sodium Salicylate in Its Higher Levels

It is shown in Table IV that the degree of inhibition by salicylate in its higher concentrations (Hh) are independent of substrate concentrations.

TABLE IV

The Relationship between the Inhibition by Sodium Salicylate in Its Higher Concentrations and the Substrate Concentrations

(The values of H in the Table are obtained by 0.274 *M* sodium salicylate).

Substrate concentration	Hh
mole/lit.	
0.01	0.64
0.005	0.58
0.0025	0.57
0.00125	0.60
0.000625	0.55

Incubation Time

Remaining activities were determined after incubation in varied length of time with sodium salicylate, the concentration of the latter being kept constant. Relation between the logarithm of the remaining activity and the incubation time could be expressed by straight lines after 10 minutes as given in Fig. 5. From this result it will be assumed that the inactivation of enzyme by sodium salicylate in its higher concentrations proceedes according to the first order kinetics. Therefore, the velocity constant k of inactivation reaction caused by sodium salicylate may be expressed as follows,

$$k=\frac{1}{t}\ln\frac{Xo}{Xt} \qquad \text{Eq. (15)}$$

where t indicates the incubation time, Xo and Xt are activities remaining after incubation for first 10 minutes and for t minutes, respectively.

The values of k in varied concentrations of sodium salicylate are shown in Table V.

The Effect of Temperature upon the Inhibition Reaction of Salicylate

From the results described above, it was concluded that there are two different

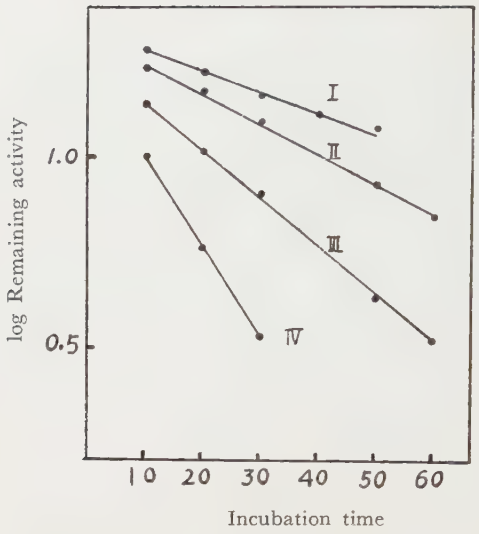

Fig. 5. Relationship between remaining enzyme activity and incubation time in the inhibition by sodium salicylate in its higher concentrations. Ordinate, logarithm of remaining enzyme activity; abscissa, incubation time in minutes. Molarity of sodium salicylate during the incubation was as follows: I, 0.230; II, 0.243; III, 0.257; IV, 0.273.

Table V

The Rate Constants of Inactivation Reaction in Various Sodium Salicylate Concentrations

(Substrate concentration: 0.0025 *M*).

Molar concentration of Na salicylate	k ($min.^{-2}$)
M	
0.230	0.0138
0.243	0.0184
0.257	0.0299
0.278	0.0553

modes of inhibition by sodium salicylate according to the difference of its concentrations applied, similarly as by urethane. Now, the temperature effects upon the degree of inhibition, H, of both types of the salicylate reaction were studied. As may be seen

in Table VI, the temperature effect is small in the inhibition by sodium salicylate in lower concentrations, while in the inhibition by higher concentrations a more pronounced temperature effect was observed.

TABLE VI

Substrate concentration: 0.0025 *M*.
H: Degree of inhibition by 0.153 *M* sodium salicylate
Hh: Degree of inhibition by 0.274 *M* sodium salicylate

Temperature	Hl	Hh
39.0°	—	0.75
37.8	0.34	0.53
34.0	0.32	0.31
30.0	0.31	0.09
25.0	—	0.09

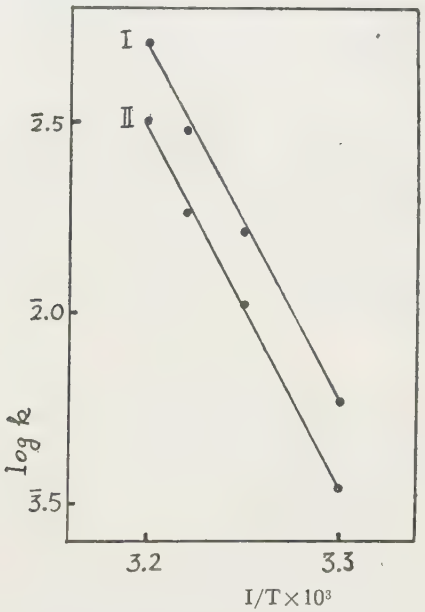

FIG. 6. The Arrhenius plots for the inactivation reaction of cholinesterase by sodium salicylate in its higher concentrations. Molarity of sodium salicylate during the incubation was as follows: I, 0.243; II, 0.257.

In these two types of salicylate inhibition, they were thus proved to be different in their reaction natures. The relation between the logarithm of the velocity constants of inactivation reaction by higher salicylate concentrations and the reciprocal of absolute temperature are shown in Fig. 6. From the slope of this straight line, the Arrhenius energy was calculated to be 42000 cal. From these results obtained, it may be inferred that the inhibition of cholinesterase by sodium salicylate in its higher concentrations is brought about through the denaturation of enzyme protein or some similar reaction, as it was suggested also in the reaction by urethane.

SUMMARY

1. Sodium salicylate inhibits the cholinesterase in two different ways as the incubated concentration of the former differs.

2. It was indicated that the treversible inhibition, which is observed in the lower concentration range of salicylate, is non competitive. The inhibition seems to be brought about by combination of two molecules of salicylate to one enzyme molecule. The binding of one molecule of substrate to one enzyme molecule may result a hindrance of the binding of salicylate molecule with the latter.

3. In the irreversible inhibition by sodium salicylate which occurs in the higher concentration of the latter, the inactivation reaction of enzyme proceeds according to the first order kinetics, and is independent of substrate concentrations. The Arrhenius energy of this reaction was calculated to be 42,000 cal.

The present investigation was supported by Prof. K. Kaziro, to whom the author's deep gratitude is due.

REFERENCES

(*1*) Shukuya, R., *J. Biochem.*, **40**, 535 (1953)
(*2*) Goldstein, A., *Arch. Biochem. Biophys.*, **33**, 22 (1951)
(*3*) Johnson, F. H., Kanzmann, W. H., and Gensler, R. L., *Arch. Biochem.*, **19**, 229 (1948)
(*4*) Kikuchi, G., and Tomimura, T., *J. Biochem.*, **41**, 503 (1954)
(*5*) Tsushima, K., *J. Biochem.*, **41**, 215, 359 (1954)
(*6*) Shukuya, R., *J. Biochem.*, **38**, 225 (1951)
(*7*) Alles, G. A., and Hawes, R C., *J. Biol. Chem.*, **133**, 375 (1950)

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STUDIES ON THE TOXICITY OF FISH OIL

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It was first depicted by Sahashi (*1*) and Somekawa (*2*) that some sorts of fish oil including whale oil are toxic to rat. But it remains undecided whether they are equally toxic to man or not. Since in Japan, where resource of fat is extremely short, the use of fish oils as nutrient is highly desirable, the wide experiments on man were carried out in many clinical as well as biochemical laboratories. The result of such cooperative works revealed that fish oil is not so toxic to man in dosage of 50–100 gms. per day. But quite recently Kaneda and Ishii (*3*) made an interesting observation that fish oil per se is not so toxic even to rat, but when it is once oxidised simply by being exposed to air, turns out to be toxic.

In this case it is well assumed that unsaturated fatty acid in fish oil is the substance easily oxidised and at the same time responsible for toxic effect. Using the unsaturated fatty acids obtained from cuttle fish oil, the author carried out the experiment and fully confirmed the results obtained by Kaneda and Ishii (*3*). Further, some chemical nature of the toxic substance was studied, the details of which are partly reported here.

EXPERIMENTAL

Preparations of Unsaturated Fatty Acids

The raw cuttle fish oil produced in Hokkaido was used. Its properties are shown in Table I.

TABLE I

The Properties Raw Cuttle Fish Oil

Density (15°)	Iodine value (Wijis)	Acid value	Sap. value	Unsap. matter (%)
0.9305	182.5	2.03	189.3	2.19

The unsaturated fatty acids were separated by the soda-acetone process (*4*). Namely, raw cuttle fish oil was saponified by ethyl alcoholic solution of NaOH, mixed with acetone, left over night at 0° and filtered. From the filtrate, which contained acetone soluble sodium-salts of highly unsaturated acids, acetone was distilled off, and its residue was decomposed by dil. HCl.

The free acids, dissolved in petroleum-ether (b. p. below 65°) were washed several times with water, and dehydrated by anhydrous sodium-sulphate and then petroleumether was distilled off, giving the highly unsaturated acids as residue.

Since the sample obtained as above, used to contain somewhat of unsaponificable matter which was existing in raw oil, so it was desirable to remove it as much as we could.

Next, the sample was dissolved in twice the volume of absolute ethyl alcohol containing 2.5 per cent HCl, and heated on the water-bath for about three hours. By this process ethyl esters of the acids were prepared. Then, the ethyl esters were fractionated under a vacuum of 2 mm. Hg at various temperatures. The 205～215° fraction was chiefly used for the author's experiment. This fraction has light yellow colour and no odour. Since these esters are liable to be rapidly oxidised when they are in contact with air, special precaution was paid throughout the preparation.

The container of the esters was always filled with CO_2 gas and was kept in dry-ice during the experiments. An aliquote portion of the sample thus prepared were put into a petri dish and borught into contact with air at the room temperature. As the oxidation proceeded the esters developed light yellow colour. At a certain point of oxidation the reaction was stopped and the ester was kept again in dry-ice avoiding further change.

Ethyl ester of oleic acid was used as control. Some chemical properties of the esters and of their oxidised compounds are indicated in Table II together with those of oleic acid ester for comparison.

TABLE II

Analysis of the Samples Used in the Experiments

	d_4^{15}	n_D^{20}	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ethylesters of highly unsaturated fatty acids	—	1.4823	358.70	163.90	Trace
Oxidised esters of highly unsaturated fatty acids	0.9568	—	264.82	201.49	Trace
Ethyl ester of oleic acid	0.8822	—	82.96	187.69	0

RESULTS

Effect on the Growth of the Rat—The rats of nearly same size and body weight

(about 60 g.) were divided into three groups, each comprising 4 rats. To the first group the feed was given containing 5 per cent of the unsaturated fatty acid esters in the basal diet, to the second group that of the oxidised esters and to the third that of oleic acid ester, which considered as control. The composition of the basal diet is given in Table III.

Table III

Ingredients of Basal Diet

Starch (rice powder)	80%	Yeast	3%
Casein (ether extracted)	9%	Liver oil	1 drop/day
McCollum salt mixture	3%		

Total amount of diet per day is 10 g. (from 1st day to 5th day), 12.5 g. (from 6th day to 10th day), 15 g. (from 11th day to the 30th day).

The experimental results are recorded in Table IV and also illustrated in the accompanying Fig. 1 and photographs (Figs. 2, 3, and 4). It is apparent that the rats fed on the unsaturated fatty acid ester containing diet showed the same satisfactory growth as those on the oleic acid ester diet. But it is quite remarkable that all rats that fed on the oxidised ester diet not only showed retarded growth but died within few days after the beginning of the experiment. It was also noticed in this group that white hair turned into brown.

Table IV

Feeding Records

Kind of ester fed	Sex	Increase of body weight (g.) 10th day	average	20th day	average	30th day	average
Ethyl ester of oleic acid.	♀	+11.0	+13.1	+34.0	+43.3	+ 61.0	+82.1
	♂	+14.0		+51.2		+113.5	
	♀	+15.0		+40.5		+ 70.8	
	♀	+12.5		+47.5		+ 83.0	
Ethyl ester of unsaturated fatty acid.	♂	+ 7.7	+ 6.3	+41.0	+38.1	+ 85.0	+75.8
	♀	+ 7.0		+38.5		+ 77.0	
	♂	+ 7.0		+44.5		+ 75.0	
	♀	+ 3.2		+28.5		+ 66.0	
Oxidised from of the above ester.	♀	−3.8 (died on 6th day)					
	♀	−5.0 (died on 7th day)					
	♀	−5.0 (died on 8th day)					
	♂	−8.5 (died on 3rd day)					

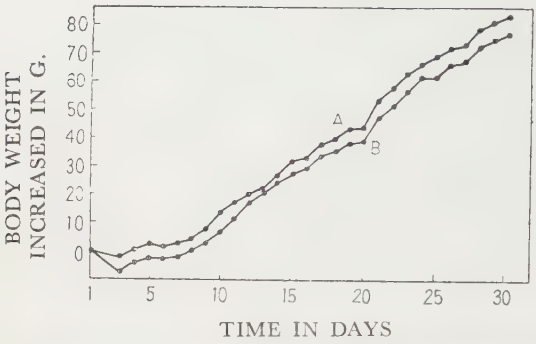

Fig. 1. The curve of average body weight increased. A (Control): average body weight increment of the rats fed on the diet containing 5 per cent of the ethyl ester of oleic acid. B: that of rats fed on the died containing 5 per cent of the ethyl ester of highly unsaturated fatty acids.

Fig. 2. The rat fed on the diet containing 5 per cent of the ethyl ester of oleic acid. (Control) On the 27th day after the commencement of the experiment.

Fig. 3. The rat fed on the diet containing 5 per cent of the ethyl ester of highly unsaturated acid. On the 27th day after the commencement of the experiment.

Effect of More Mild and Chronic Intoxication by the Oxidised Ester—For this purpose the grown-up rats were fed on the diet containing 5 per cent of the oxidised ester not daily but at some intervals.

One of the typical examples is illustrated in Fig. 5 with regard to the change of body weight.

As shown in Fig. 5, the retarted growth of the animal continued during the feeding of the toxic diet, but somewhat recovering was observed when that feeding was stopped. It ended, however, in fatal at the end of 23th day after the commencement

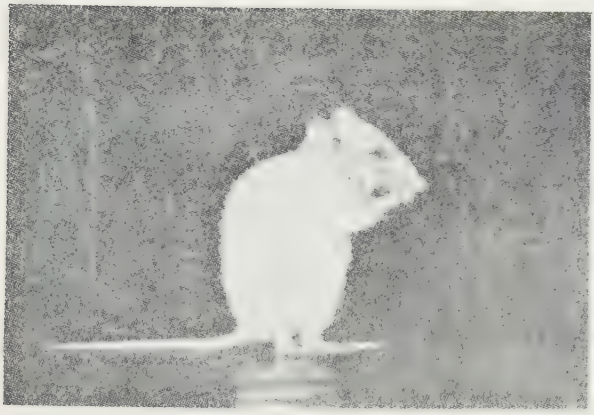

Fig. 4. The rat fed on the diet containing 5 per cent of the oxidised ethyl ester of highly unsaturated acid. On the 4th day after the commencement of the experiment.

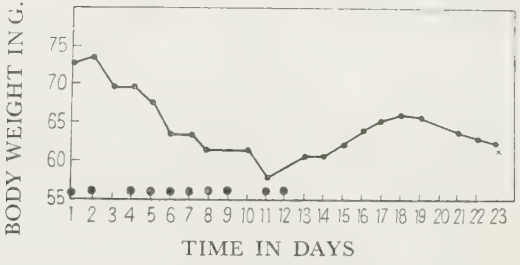

Fig. 5. The curve of body weight changed.
The mark shows the day on which the oxidised ester diet was given.

of the experiment. The appearance of the animal was quite particular. The hair of the face was diplated off, the mouth and rear legs were swollen, the remaining hair changed into brown, and hemorrhages were seen here and there in the skin. (see Figs. 6 and 7).

DISCUSSION

The above results substantially proved the finding first made by Kaneda and Ishii that the highly unsaturated acid from fish oil is not toxic but rather its oxidised form is toxic. As it is usually assumed that unsaturated fatty acid absorbs oxygen forming peroxide at double bonds in the molecule, so it might be plausible that the toxicity of the oxidised acid is caused by peroxide. In this regard the next paper will give full details. At any rate it is promising that fish oil can be utilised

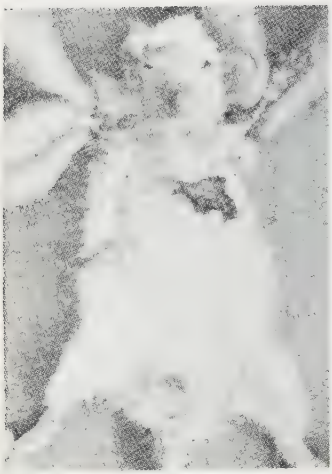

FIG. 6

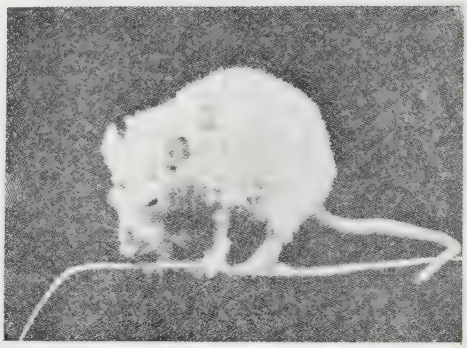

FIG. 7

as human nutrient when it is prepared and preserved avoiding spontaneous oxidation. Or it may be more safe to hydrogenate fish oil to certain extent to get rid of double bonds.

Besides such important problems of fish oil as fat resources in nutrition, the essential feature of toxic effect of the oxidised unsaturated fatty acid from fish oil is interesting to investigate. In this line the work of Kaunitz and Johnson (*5*) is noteworthy. They observed that rat fell into flavin-shortage when rancid lard was given. But it seems to the author that the toxicity is too acute to ascribe it solely to riboflavin shortage.

SUMMARY

1. When the diet containing 5 per cent ethyl esters of highly unsaturated fatty acids obtained from cuttle fish oil was given to rat, it showed quite the same good nutritive effect as the diet containing oleic acid ester.

2. To the contrary the oxidised form of the above unsaturated fatty acid esters showed an extremely toxic effect to rat.

REFERENCES

(*1*) Sahashi, Y., *Sci. Pap. Inst. Phys. Chem. Research (Tokyo)* **20**, 245

(1933)

(2) Somekawa, E., *Sci. Pap. Inst. Phys. Chem. Research (Tokyo)*, **20**, 149 (1933)

(3) Kaneda, T., and Ishii, S., *Bull, Jap. Soc. Sci. Fisheries*, **19**, 171 (1953)

(4) Toyama, S., and Tsuchiya, T., *J. Soc. Chem. Ind. Japan*, **28**, 963 (1925)

(5) Kaunitz, H., Johnson, R. E., and Slanetz, C. A., *J. Nutr.*, **46**, 151 (1952)

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STUDIES ON THE METABOLISM OF THE MYXOMYCETE PLASMODIUM

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The plasmodium of the Myxomycete, commonly known as a slime mould, is a huge mass of naked protoplasm without any cell wall. It grows to such a big mass that it is not only visible to the unaided eye, but easily can be collected in quantity required for the experiment. The plasmodium consists of outer plasmagel and of inner plasmasol. The inner plasmasol shows active streaming, which reverses its direction alternately with a definite period. Because of these characteristics, the plasmodium has been used as a favourable material for studies on various physiological activities of protoplasm, especially on protoplasmic streaming.

The effects of various respiratory poisons on the generation of the motive force responsible for the protoplasmic streaming in the plasmodium were investigated by the author (*1*) and Kamiya, Nakajima and Abe (*2*). To analyse the phenomena causally, however, it is important to be informed of the metabolic character of this organism. Since Allen and Price (*3*) recently reported respiratory features of the plasmodium in considerable detail, the present work was carried out in order to obtain further information on the metabolism of this organism including fermentation.

MATERIALS AND METHODS

In the present work, the plasmodium of *Physarum polycephalum* was used as material. It was cultivated with rolled oats, according to the method of Camp (*4*).

From the advancing fronts of the plasmodium, which are almost free from bacteria and protozoa, appropriate amounts (about 50 mg.) of protoplasm were taken off. Samples thus separated were freed from excessive water coating them, before they were weighed by torsion balance. They were then transfered into manometer vessels.

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O_2 consumption and CO_2 production were measured by Warburg manometers. Solutions in the vessels were buffered at pH 6.0 with *M*/45 Sörensen's phosphate buffer except as otherwise indicated. Experiments were made at 25±0.5°.

As the amounts of respiration of the plasmodium showed small deviations from one sample to another, control reading was taken before inhibitors or substrates were added to the material from side arms. When O_2 uptake was measured under the presence of cyanide, Krebs' KCN-KOH mixtures were used in place of KOH. Amounts of total nitrogen in the plasmodium were determined by the micro-Kjeldahl method.

RESULTS

Aerobic Metabolism

General Features—In a medium of *M*/45 phosphate buffer at pH 6.0, the plasmodium respired with Q_{O2} of 1.08 with the standard deviation (from 52 data) of 0.16 on a wet weight basis. On a dry weight basis and on a total nitrogen basis, the corresponding Q_{O2} values are 7.3 and 140, respectively.

The respiration continued at an almost linear rate at least for 5 hours. Amounts of O_2 uptake are more or less variable according to the sample, but plasmodia originating from the same stock culture show much less deviations in Q_{O2} values. This deviation is nearly the same in amount as to the deviation of the total nitrogen content of the specimen from the same stock culture.

Allen and Price (*3*) reported a dependence of Q_{O2} values on the weight of the specimen. According to them, maximum O_2 consumption per unit amount was reached when the material weighed 50 mg. We could not find any evidence supporting their statement.

R.Q. values averaging at 0.83, showed only insignificant fluctuations. When plasmodia were not fed for 2 days, Q_{O2} value lowered to about 80 per cent of that under the well fed state. In this case, however, the R.Q. value remained unchanged.

Effects of pH—Respiration is affected not only by the pH, but also by the concentration of the buffer, as shown in Table I. The effect of the concentration of the buffer was not due to its osmotic pressure, because O_2 uptake suffered no inhibition in the media of *M*/10 and *M*/15 mannitol. Therefore, it is likely that high concentrations of the buffers used bring about the decrease of respiration.

The plasmodium showed almost a constant rate of respiration unless the pH is reduced below 4.0, or raised above 7.0. When the pH was reduced to 3.0, the colour of plasmodium became golden yellow and its microscopic structure was disintegrated within 15~30 minutes. In contrast to this, plasmodium tended to be decolourized in the medium of pH 7.0 and above.

Effects of Inhibitors—

(1) KCN: The respiration of the plasmodium was very sensitive to cyanide. An appreciable inhibition was observed even at a concentration of 5×10^{-4} *M*. How-

TABLE I

Relation between the pH of the External Medium and Oxygen Consumption

	Buffers used	pH						
		3.0	4.0	5.0	5.2	6.0	7.0	8.0
O_2 uptake in per cent of control in distilled water	1/4 concentration of McIlvaine's citrate buffer	8	79.5	75.0		67.0	27.7	13.5
	1/8 concentration of McIlvaine's citrate buffer	61.1	101	103		106	83.3	75.0
	M/20 Sörensen's phosphate buffer				103	104	77.5	25.2
	M/40 Sörensen's phosphate buffer				102	103	98.0	77.3

ever, about 20 per cent of O_2 uptake remained when concentrations of KCN were raised even above 10^{-2} *M* (Fig. 1).

(2) CO: By replacing the air in the manometer vessels with 95 per cent CO+5 per cent O_2, the O_2 uptake considerably decreased. Such decrease was not observed in the gas mixtures of 95 per cent N_2+5 per cent O_2. The degree of inhibition by CO was variable, depending on the condition of the plasmodium. Freshly fed material suffered more severe effects than starved one. CO inhibition recovered to some extent upon illumination (Table II).

(3) NaN_3: Sodium azide severely inhibited the respiration at concentrations above 5×10^{-4} *M*. However, at lower concentrations of this inhibitor (5×10^{-5} *M*), slight increase of O_2 uptake was observed (Fig. 2).

(4) 2,4-Dinitrophenol: 2,4-Dinitrophenol stimulated respiration by about 40 per cent of the control at 5×10^{-5} *M*., but it inhibited the O_2 uptake at concentrations above 5×10^{-4} *M*. (Fig. 3)

(5) CH_2ICOOH: The O_2 uptake of the plasmodium was also inhibited by monoiodoacetate, as shown in Fig. 4. As the degree of this inhibition was considerably high, it seems probable that glycolysis takes part in the respiratory process of this organism.

(6) Other inhibitors: Slight decrease of respiration was observed in the presence of high concentration of sodium fluoride, while malonate and inhibitors of polyphenol oxidase (phenylthiourea and thiourea) excerted no marked inhibition of respiration. Effects of these inhibitors were summarized in Table III.

Effects of Extracellular Substrates—In order to obtain more precise information concerning the individual enzyme system in this plasmodium, effects of added substrates were investigated. However, no useful results to elucidate the situation

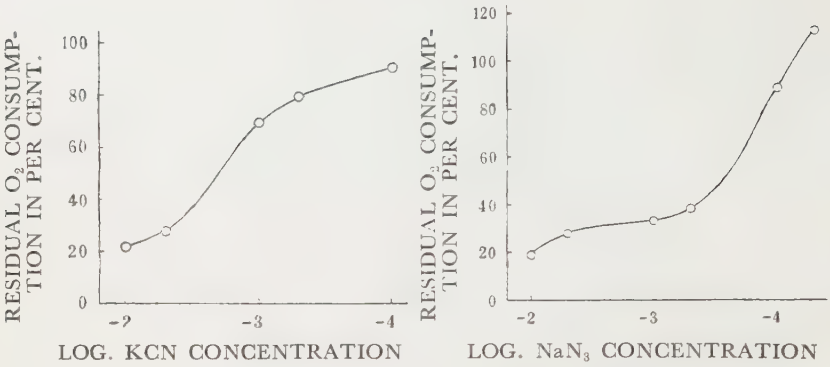


Fig. 1. Residual respiration of plasmodium at different concentrations of cyanide, as percentage of the control rate before addition of cyanide.

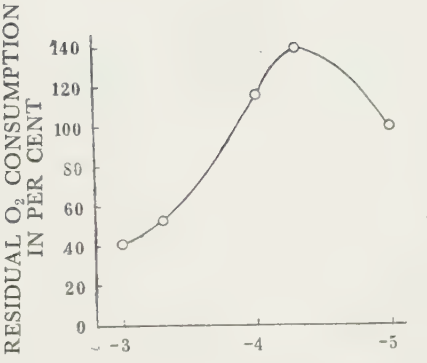
Fig. 2. Residual respiration of plasmodium at different concentrations of azide, as percentage of the control rate before addition of azide.

Table II

Effects of Illumination on the CO Inhibition

	Exp. No.	Dark	Light	Dark	Light
Residual O_2 uptake in per cent of control	1	38.0	52.7	40.8	54.0
	2	52.1	62.3	37.8	63.0
	3	41.8	49.4	43.7	49.8
	4	41.7	42.9	41.4	43.0
	mean	43.4	51.8	40.9	52.5

were obtained, because almost all the substrates induced no marked increase of respiration as shown in Table IV. Glucose slightly increased O_2 consumption. This is also true to a lesser extent for acetate and formate. But, it is uncertain whether or not these substrates were really metabolized. Propionate and butyrate decreased respiration strikingly at a concentration of 10^{-2} *M*, where the surface of the plasmodium was broken, and its protoplasmic structure was disintegrated. Since the concentration inducing no changes in visible structure (10^{-3} *M*), has no effect on respiration, it seemed improbable that these fatty acids act as enzyme poisons.



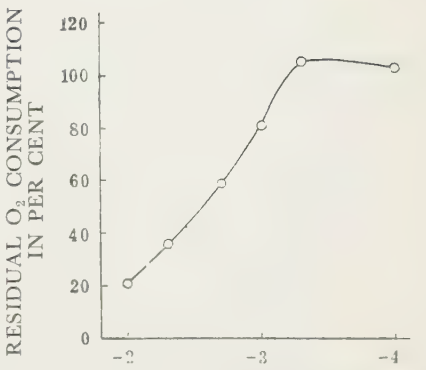


FIG. 3. Residual respiration of plasmodium at different concentrations of 2,4-dinitrophenol, as percentage of the control rate before addition of dinitrophenol.

FIG. 4. Residual respiration of plasmodium at different concentrations of monoiodoacetate, as percentage of the control rate before addition of monoiodoacetate.

TABLE III

Effects of Some Inhibitiors on Respiration

Inhibitors	Concentration	Respiration (per cent of control)
Sodium fluoride	10^{-2} *M*	84.5%
Sodium fluoride	5×10^{-3}	95.1
Sodium fluoride	10^{-3}	103
Sodium fluoride	10^{-4}	102
Sodium malonate	10^{-2}	101
Sodium malonate	10^{-3}	104
Phenylthiourea	10^{-3}	87.0
Phenylthiourea	5×10^{-4}	89.2
Phenylthiourea	10^{-4}	99.0
Thiourea	10^{-3}	103
Thiourea	10^{-4}	101

Detection of Cytochrome System—As already mentioned, cyanide, azide and CO considerably inhibited the O_2 uptake of the plasmodium. Therefore, it is likely that

TABLE IV

Effects of Extracellular Substrates on Respiration

Substrates added	Concentration	Respiration (per cent of control)
Glucose	10^{-2} *M*	136%
Sodium citrate	10^{-3}	102
Sodium succinate	10^{-3}	104
Sodium malate	10^{-4}	102
Sodium fumarate	10^{-2}	101
Sodium pyruvate	10^{-3}	103
Sodium acetate	10^{-3}	121
Sodium propionate	14^{-4}	15.0
Sodium propionate	10^{-4}	101
Sodium butyrate	10^{-3}	12.9
Sodium butyrate	10^{-4}	103
Sodium formate	10^{-3}	116
Sodium lactate	10^{-4}	104

cytochrome system participates in the oxidative process of this organism. This presumption was verified by the following results: The presence of cytochromes *a*, *b* and *c* in healthy plasmodia was found spectroscopically, and it was observed that macerated plasmodium rapidly reduced indophenol-blue from Nadi reagent. The latter fact has already been reported by Allen and Price (*3*).

The role of polyphenol oxidase in respiratory process of this organism, seems not important, because no significant effects were observed by the addition of inhibitors of this enzyme, as already shown.

From these facts, it can be concluded that the cytochrome oxidase is a terminal oxidase in the respiration of this plasmodium.

Anaerobic Metabolism

Amounts of anaerobic CO_2 production were very little, if at all. The $Q_{CO_2}^{N_2}$ (based on wet weight) averaged 0.11 from 15 data. This value corresponds to about one eighth of that of aerobic CO_2 production. By the addition of glucose, no increase of aerobic CO_2 production was observed.

A considerable amount of acid or acids was produced by the plasmodium under anaerobic condition. Mean $Q_{acid}^{N_2}$ (on a wet weight basis) is 0.514, as compared with Q_{acid}^{air} (aerobic acid formation) of 0.083. What acid or acids were produced, has not been determined.

By comparing the value of $Q_{Co_2}^{N_2}$ with that of $Q_{acid}^{N_2}$, it seems to the author

that the principal fermentative process in this organism is not an alcoholic fermentation, but a fermentation which is accompanied by acid formation.

In addition to this, it can be considered that no aerobic fermentation participates in the metabolic process of the plasmodium, because the amount of aerobic acid formation is negligible, and R. Q. value is 0.83.

From the facts mentioned above, it is certain that the "Pasteur reaction" takes place when the plasmodium which has been kept under anaerobic condition is transferred to aerobic condition.

DISCUSSION

Since Kamiya (*5*) had deviced the method for measuring the motive force of protoplasmic streaming in plasmodia of *Physarum polycephalum*, several investigations have been reported in regard to the nature of the motive force. The author (*1*) showed that the motive force did not decrease, but rather increased to a slight extent under anaerobic condition and in the presence of respiratory poisons such as cyanide and CO. On the other hand, Kamiya *et al.* (*2*) found that agents which were known as inhibitors of fermentation (monoiodoacetate and sodium fluoride), induced marked decrease of the motive force. They also indicated that the motive force was reversibly inhibited by 2,4-dinitrophenol, and that the increase of the motive force was induced by the addition of adenosine triphosphate to the plasmodium.

These facts suggest as pointed out by Kamiya *et al.* (*2*), that the energy supply for protoplasmic streaming comes from the energy rich phosphates which are produced in the process of fermentation. In this respect the protoplasmic streaming of the plasmodium seems to be similar to the muscular contraction. Another similarity between these two mechanical activities, was reported by Loewy (*6*) who suggested the presnce of an actomyosine-like structural protein in this plasmodium.

In this paper, the author reported that the metabolism in this plasmodium also resembled that of muscular tissues, in the following respects: (1) The fermentation of the plasmodium is accompanied by the production of acid, which is observed only under anaerobic condition; (2) Sensitivity of respiration of the plasmodium to various inhibitors, especially to cyanide, CO and monoiodoacetate, coincides with that of the respiration of muscular tissues to the same inhibitors; (3) As monoiodoacetate severely inhibited respiration, it seems probable that glycolysis takes part in the metabolism of the plasmodium; (4) The presence of cytochromes *a*, *b* and *c* in this organism was confirmed; (5) The

terminal oxidase of respiration in the plasmodium is cytochrome oxidase.

Plasmodia of slime moulds and muscular tissues of higher animals both perform mechanical work, though the latter is far more differentiated than the former. It is worth noticing that also in metabolic aspects, there are many common characteristics between the two.

In conclusion, the author desires sincerely to acknowledge his indebtedness to Prof. K. Okunuki and to Prof. N. Kamiya for their kind guidance and help throughout this investigation. The author also wishes to express his gratitude for the financial aids from the Yukawa Fellowship of Osaka University.

SUMMARY

The metabolism of the Myxomycete plasmodium (*Physarum polycephalum*) was investigated by using Warburg manometers. The following results were obtained.

1. Q_{02} value on a wet weight basis was 1.08 ($M/45$ phosphate buffer at pH 6.0), which was 7.3 on a dry weight basis and 140 on a total nitrogen basis.

2. R. Q. value was found to be 0.83 in average.

3. Respiration was affected not only by the pH, but also by the concentration of the buffer. From pH 4.0 to pH 7.0, the respiratory rate was almost constant.

4. O_2 uptake was strongly inhibited by various metabolic poisons such as cyanide, NaN_3 and monoiodoacetate. CO inhibited respiration considerably, but the inhibition was reversed to some extent upon illumination. Effects of the other inhibitors (2,4-dinitrophenol, NaF, malonate, phenylthiourea and thiourea) were also investigated. Considering the effects of these inhibitors, the terminal oxidase in the respiration of the plasmodium is suggested to be the cytochrome oxidase. Further support of this was afforded by the spectroscopic confirmation of cytochromes *a*, *b* and *c*.

5. No marked increase of O_2 consumption was brought about by the addition of various substrates.

6. Under anaerobic condition, the plasmodium showed the fermentation which was accompanied by acid formation. No sign of aerobic fermentation was observed.

These results show that the metabolism of the plasmodium is very similar to that of muscular tissues.

REFERENCES

(1) Ohta, J., *Cytologia*, **17**, 300 (1952)
(2) Kamiya, N., Nakajima, H., and Abe, S., *Seitaino-kagaku* (Medical Science) (in Japanese), **5**, 148 (1954)
(3) Allen, P. J., and Price, W. H., *Amer. J. Bot.*, **37**, 393 (1950)
(4) Camp, W. G., *Bull. Torrey Bot. Club*, **63**, 205 (1936)
(5) Kamiya, N., in *The structure of protoplasm* (edited by Seifriz) Monograph Amer. Soc. Plant Physiol. (Ames-Iowa), 199 (1942)
(6) Loewy, A. G., *J. Cell. Comp. Physiol.*, **40**, 127 (1952)

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STUDIES ON FERRITIN

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(Received for publication, April 6, 1954)

As ferritin can be obtained in chemically pure crystalline form, numerous experiments have been carried out on its physicochemical properties. However, a problem of fundamental importance, namely whether the iron atom is inside or outside the ferritin molecule, has not yet been worked out. The present work is concerned with the acid and alkali titration of ferritin and apoferritin to examine the dissociation of H^+ or OH^- from the molecule, with the view of obtaining information concerning the state of iron bound by apoferritin to form ferritin.

EXPERIMENTAL

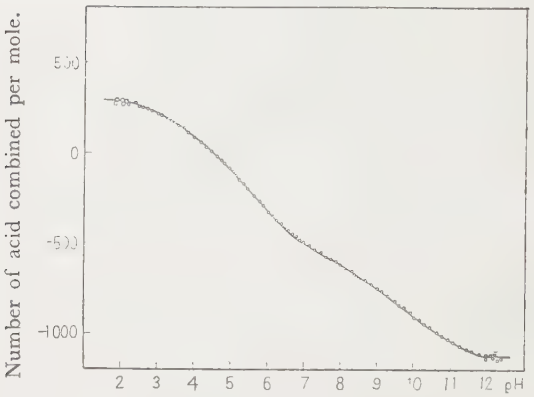
Ferritin and apoferritin were prepared from horse spleen by the Granick method (*1*), recrystallized four times from cadmium sulfate and found to be electrophoretically homogeneous. According to Granick (*1*), molecular weight of apoferritin is 460,000 and its isoelectric point, 4.4. Titrations were carried out at a very low ionic strength, with hydrochloric acid and sodium hydroxide, in a range of pH 2–12. For the titration, to 25 ml. of ferritin solution, hydrochloric acid were added to adjust its pH to 2.0. After complete removal of carbon dioxide, the solution was titrated with 0.1 *N* or 1 *N* sodium hydroxide. The protein concentration at the time of titration was determined by the micro-Kjeldahl method and titration was carried out at $2\text{–}5\times10\text{–}6$ *mole/lit.* of ferritin or apoferritin, the nitrogen content of apoferritin being taken as 16.1 per cent (*1*). Determination of pH was made in an oil thermostat with Beckman's glass electrode adjusted with Clark's buffer (*2*).

The amount of hydrogen or hydroxyl ions that was bound by protein is the difference between the amount of H^+ or OH^- added and that remaining in the solution. Activity coefficients of H^+ and OH^- necessary for the above calculation were taken from the Chemical Handbook (*3*). The iron content of ferritin and of apoferritin was found to be 21 and 2 per cent, respectively, by the *o*-phenanthroline method.

RESULTS AND DISCUSSIONS

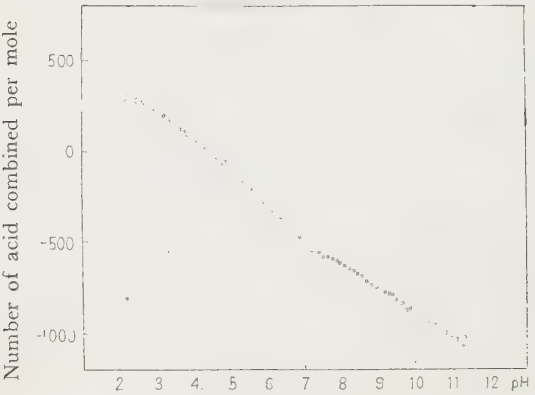
By plotting the amount of hydrogen ions bound on the ordinate

Fig. 1. Ferritin, 25°C



The acid combining capacity of ferritin at 25°C.
oooo observed
—— calculated

Fig. 2. Ferritin, 10°C

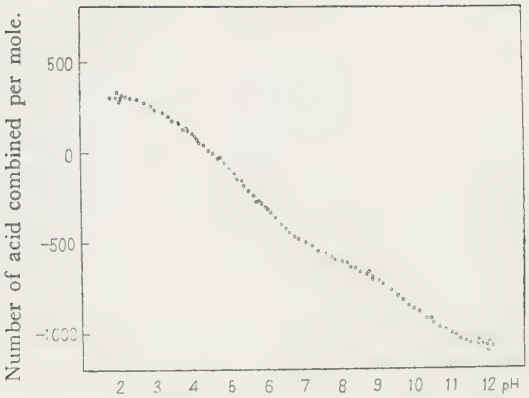


The acid combining capacity of ferritin at 10°C

and pH on the abscissa, the dotted lines in Figs. 1–3 were obtained. As apoferritin forms reversible precipitate near its isoelectric point at 25°, the results of titration shown are those obtained at 10°. Titrations of ferritin were carried out at 10° and 25°.

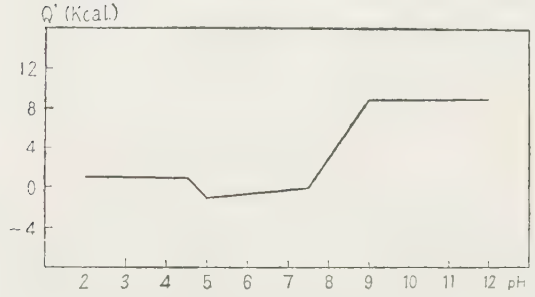
Analysis of titration of ferritin at 25°, by breaking down to the smallest possible number of dissociation constants, showed that the

FIG. 3. Apoferritin, 10°C



The acid combining capacity of apoferritin at 10°C

FIG. 4



Apparent heat of dissociation, Q', in the 10°~25°C, plotted against pH

results can best be explained by assuming the following pK groups and numbers for them.

pK	3.8	5.7	6.7	7.9	9.2	9.9	11.0
Number	350±20	360	130	150	160	100	180±30
			±10				

The curve in Fig. 1 was drawn on this hypothetical basis and agrees well with the measured values. Apparent heat of dissociation, Q', obtained from the titration of ferritin at 10° and 25° according to Wyman (*4*), is shown in Fig. 4, and the buffering action seen from

strongly acid to neutral range is assumed to be due to carboxyl groups, from which it may be said that ferritin possesses a large number of carboxyl groups of weak acidity.

The curve is almost the same in the case of apoferritin, even if a few thousand atoms of iron are detached from one molecule, there would be no effect on the dissociation of dissociating groups.

The data obtained by the present experiments agree with the results of electrophoretic mobility of ferritin and apoferritin obtained by Mazur and Shorr (*5*), and mobility-pH curve obtained by them shows a form that is similar to the titration curve obtained in the present experiments. However, no clue has been obtained as to how iron is attached in ferritin.

SUMMARY

Acid-base titrations were carried out on ferritin and apoferritin obtained from horse spleen, and analyses of the dissociating groups in the molecule were made on the basis of the titration data.

The authors express their cordial thanks to Prof. H. Tamiya, Prof. N. Shimazono, Dr. Y. Ogura, and Dr. M. Yoneyama for their kind guidance.

REFERENCES

(*1*) Granick, S., *Chem. Rev.*, **38**, 379 (1946)
(*2*) Clark, W. M., The determination of hydrogen ions, 3rd Ed. (1928)
(*3*) Kieland, J., J.A.C.S. **59**, 1675 (1937)
(*4*) Wyman, J., *J. Biol. Chem.*, **127**, 1 (1939)
(*5*) Mazur, A., and Shorr. E., *J. Biol. Chem.*, **182**, 607 (1950)

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INTERRELATION BETWEEN THE FUNCTION OF HEMEPROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

III. EFFECTS OF SODIUM SALICYLATE AND BENZOATE UPON THE REACTION OF CHOLEGLOBIN FORMATION

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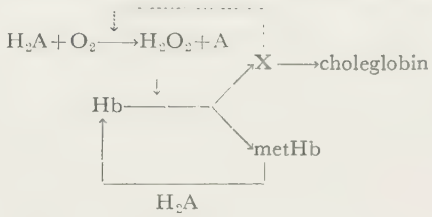
(Received for publication, April 17, 1954)

For the reaction process of choleglobin formation in the system of hemoglobin—ascorbic acid—oxygen, a reaction scheme has been presented (*1*) on the basis of our experimental evidences as follows:



where H_2A is ascorbic acid.

In the above scheme, an intermediate compound x was introduced as a presumption. The intermediate x was postulated to be a hemoglobin derivative which arises from native hemoglobin through a slight grade of denaturation in its globin part. The intermediate may be much more active as an oxidative catalyst than the native hemoglobin, thus being responsible for the chain reaction nature of the process of choleglobin formation.

On the other hand, Tsushima (*2*) in our laboratory reported recently on his detailed studies regarding a slight and reversible denaturation of various hemoglobin derivatives, such as reduced Hb, metHb and HbO_2, which was caused by the action of sodium salicylate and benzoate, respectively. The denaturation in these cases were proved to be completely reversible in the meaning of Holden's "perturbation". It is of special interest in Tsushima's paper, that the autoxidation of HbO_2 was also proved to be accelerated significantly by those

"perturbators". Holden (*3*) reported in 1947, that a series of reagents being able to perturb hemoglobin under suitable conditions, promoted the formation of green pigment; this was observed by repeated desoxygenation and reoxygenation of horse HbO_2 by $Na_2S_2O_4$ and by atmospheric oxygen alternatively, or by the addition of H_2O_2 in the presence of $Na_2S_2O_4$. In Holden's paper, a suggestful quotation was made as follows: "Since a lower concentration of a perturbator than is required for a visible degree of perturbation is adequate for the promotion of green pigment formation, one is tempted to assume an intermediate step whereby a little of the hemoglobin is modified in such a way that the molecule oxygen neither unites with iron atom nor so reacts as to denature the protein, but takes a third course".

His assumption, however, has not heretofore been susceptible of experimental verification, since neither the nature of the "perturbation" of hemoglobin molecule nor its relation to the process of green pigment formation could be ascertained. His observations were only qualitative.

The present work was undertaken with the aim of determining the underlying relationship between the structural and functional modifications of hemoglobin molecule induced by salicylate and benzoate, respectively, with special references to their influences upon the reaction rate of choleglobin formation.

EXPERIMENTAL METHODS

Crystalline equine and bovine HbO_2 for our experimental purpose were prepared following the method of Heidelberger (*4*) improved in some points by Suzuki *et al.* (*5*). The samples contained neither catalase nor metHb in their fresh state. Crystalline HbO_2 was dissolved in redistilled water as a stock solution. This was diluted to 1.5×10^{-4} *M* of heme concentration in each time at use.

Procedure—Into small beakers are placed 2.5 ml. of HbO_2 solutions, 1.0 ml. of sodium salicylate or benzoate solution in various concentrations were added and were incubated at 37° for 15 minutes. Then, 0.5 ml. of 0.8 *M* ascorbic acid neutralized with equivalent NaOH was added into each of the beakers and were kept standing at 37° so as to be reacted by atmospheric oxygen. The reaction was stopped by the addition of NaOH at each interval of time. The reaction solution was then reduced by addition of a small amount of powdered $Na_2S_2O_4$ and the choleglobin in the solution was measured spectrophotometrically. The readings were made by a Hitachi photoelectric spectrophotometer. The amount of choleheme formed was calculated following the procedure described in our previous paper (*1*) according to the formulations presented in the same place. Oxygen uptake mainly for the ascorbic acid oxidation was measured manometrically by Warburg's technique.

RESULTS

Experiments with Sodium Salicylate—The reaction rate of choleglobin formation, as well as the final values of choleheme formed, increased with increasing concentration of salicylate unless it exceeded over a certain limit. When, however, the salicylate concentration exceeded this limit, the reaction rate decreased and the final amount of choleheme formed remained far below its maximum value (Fig. 1). The same

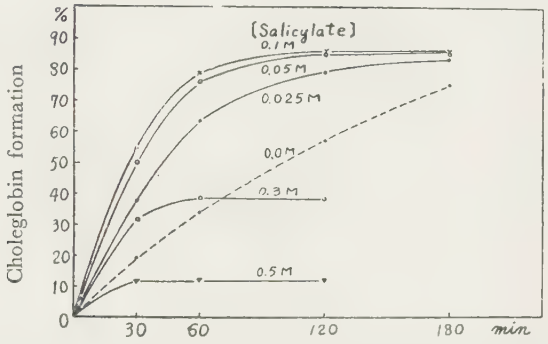


FIG. 1. Choleglobin formation in the presence of sodium salicylate. 2.5 ml. of horse HbO_2 (1.5×10^{-4} *M* as the heme concentration), 1.0 ml. of salicylate of varied concentration, 0.5 ml. of 8×10^{-2} *M* ascorbic acid. T=37°

The numbers designated in the figure represent the final concentrations of salicylate in the reaction mixture.

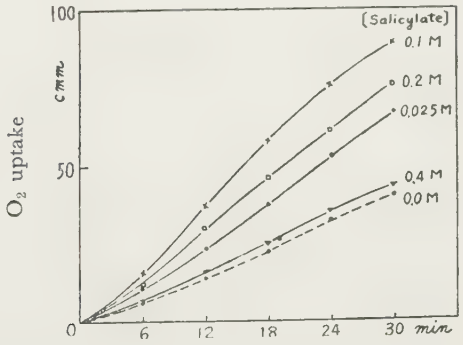


FIG. 2. O_2 uptake in the presence of sodium salicylate. Composition of the reaction mixture and temperature were the same as described in Fig. 1.

was found in the O_2 uptake in the same reaction series (Fig. 2). The effect of salicylate varying in concentrations upon the present reaction can be expressed by the changes in the reaction rate in percentages to that of the control experiments without salicylate. Both the rate of choleglobin formation and that of O_2 uptake reached the maximum value at the concentrations of salicylate within 0.05 to 0.15 *M*. Within this range of concentrations the rates were found to be constant indicating a distinct plateau in each of the curves in Fig. 3 and 4. The same was observed also in the experiment with bovine HbO_2 but rather within wider concentration range of 0.05 to 2.0 *M*.

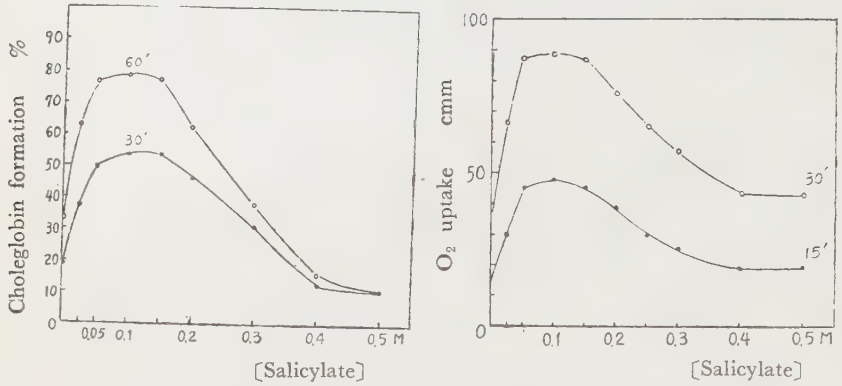


Fig. 3. Effect of sodium salicylate added in various concentrations upon the reaction rate of choleglobin formation (derived from Fig. 1). Comparison was made with the values of choleglobin formed during 30 and 60 minutes of the reaction.

Fig. 4. Effect of sodium salicylate added in various concentrations upon the rate of O_2 consumption (derived from Fig. 2).

Comparison was made with the values of O_2 uptake during 15 and 30 minutes of the reaction.

Experiments with Sodium Benzoate—The effect of benzoate upon the reaction of choleglobin formation was proved to be similar to that of salicylate, as shown by the Curves II and II' in Fig. 5. For the promotion of choleglobin formation, higher concentration was needed of benzoate than of salicylate. The range of benzoate concentrations within which a maximum and a constant promotion of the rate was observable, was found to be much wider than in the case of salicylate. In these cases with benzoate, no appreciable difference in the reaction could be proved between bovine HbO_2 and equine HbO_2.

Effect of the Prolonged Incubation of HbO with the Perturbators—The perturbation of hemoglobin proceeds with time as reported by Tsushima (*2*). Thus, it seems to be of some interest to investigate the effect of prolonged incubation of HbO_2 with

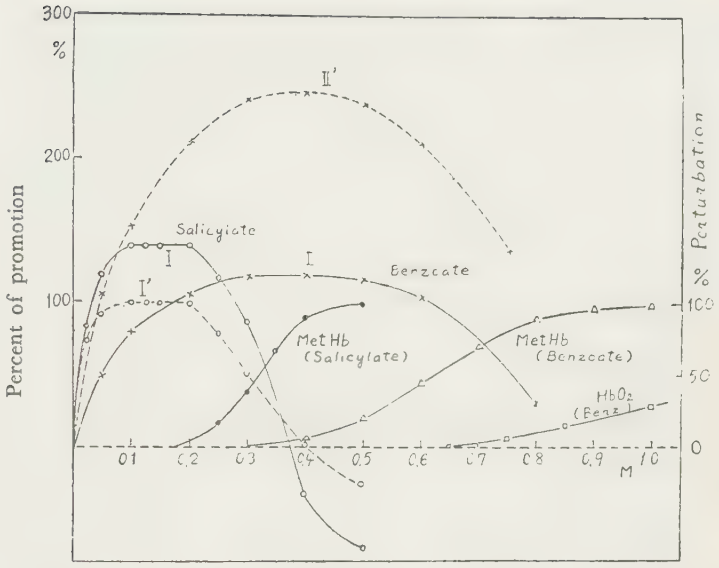


FIG. 5. Percentage representation of the effects of salicylate and benzoate upon the reaction of choleglobin formation. Bovine hemoglobin was used in these series of experiments.

I. Effects of salicylate upon the choleglobin formation.
I'. Effects of salicylate upon the O_2 consumption.
II. Effects of benzoate upon the choleglobin formation.
II'. Effects of benzoate upon the O_2 consumption.

The curves without denotation of numbers represent:

—●—●— Per cent of the visible degree of perturbation of metHb by sodium salicylate.
—△—△— The same by sodium benzoate.
—□—□— Per cent promotion of tha autoxidation of HbO_2 by benzoate.

salicylate upon the choleglobin formation. For the purpose, HbO_2 was first incubated with salicylate for various lengths of time and thereafter the reaction of choleglobin formation was started by addition of ascorbic acid. With the concentrations of 0.125 *M* of salicylate, no appreciable effect of incubation time was recognized upon the choleglobin formation, while in the salicylate concentration of 0.25 *M*, both the rate of the reaction and the final yield of choleglobin decreased according to the length of incubation time (Fig. 6).

Effect of the Temperature of Incubation—The effect of temperature upon the reaction of choleglobin formation in the presence of salicylate or benzoate was very

significant and characteristic. The temperature effect on the reaction rate was much greater in the presence of salicylate or benzoate than without them (Figs. 7 and 8). While at ralatively lower temperatures, the formation of choleglobin increased with increasing concentrations of salicylate or benzoate; at higher temperatures, however, the maximum rate was observed only within a definite concentration range of perturbator. The optimum shifted gradually toward the lower concentrations of perturbators as the temperature became higher. These facts can only be plausibly explained by the concept that the perturbation is a sort of protein denaturation.

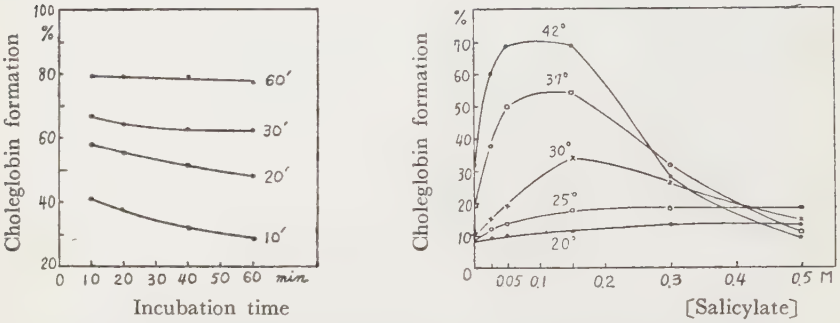


Fig. 6. Effect of the incubation time upon the choleglobin formation. Concentration of salicylate in the reaction mixture was 0.25*M*. The numbers in the figure represent the time in minutes after the reactions of choleglobin formation started on addition of ascorbic acid.

Fig. 7. Effect of the temperature upon the choleglobin formation in the presence of salicylate in various concentrations.

Comparison was made with the values of choleglobin formed during 30 minutes of the reaction.

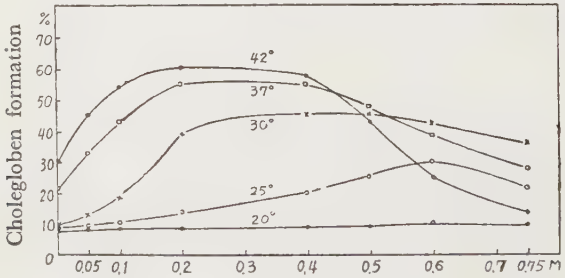


Fig. 8. Effect of the temperature upon the choleglobin formation in the presence of benzoate in various concentration.

Comparison was made with the values of choleglobin formed during 30 minutes of the reaction.

Reaction Taking Place after the Visible Degree of Perturbation has been in Completion—Bovine methemoglobin was used in this reaction series. The concentrations of salicylate and benzoate were kept highly enough to bring about the visible degree of perturbation in completion within the incubation of 15 minutes. The results are shown in Figs. 9 and 10. In cases with salicylate, the rates of both the choleglobin formation and the O_2 uptake were very small, while in cases with benzoate the reaction proceeded steadily with time, the rate of O_2 uptake being found to be greater than that of control experiment.

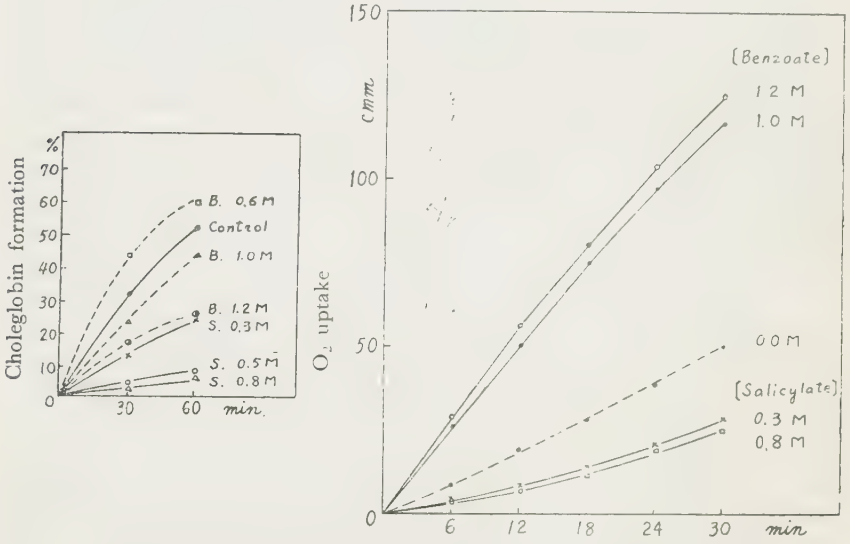


Fig. 9. Choleglobin formation in the presence of sufficiently high concentration of salicylate or benzoate as enough to bring about the perturbation of visible degree of hemoglobin. Bovine metHb was used in this series of experiments. Other reaction conditions were the same as described in Fig. 1.

Fig. 10. O_2 uptake in the same reaction conditions as described in Fig. 9.

DISCUSSION

From the results presented here, it is evident that salicylate and benzoate promote the oxidative activity of hemoglobin as well as the decomposition of the latter into choleglobin in the presence of ascorbic acid as the substrate. A mild denaturation of protein part affected by

salicylate or benzoate may be thus responsible for the promotion of oxidative activity of hemoglobin molecule. And when the protein part is affected one step further, an inactivation takes place instead. The same conclusion can be drawn from the results of experiments with longer incubation at various reaction temperatures. This sort of stepwise modification by these denaturants according to their concentration gradient could also be observed by Shukuya (*6*) and Tsushima (*2*). In Fig. 5, the results of Tsushima (*2*) indicating the effective concentrations of salicylate and benzoate for the perturbation of methemoglobin and for the promotion of HbO_2 autoxidation were additionally given beside our results. As seen from the figure, the concentrations of salicylate and benzoate favoring the promotion of choleglobin formation were found to be lower than that required for the spectral changes of methemoglobin into hemichrome and were far lower than those demanded for the promotion of HbO_2 autoxidation. These facts seem to be suggesting clearly the possibility of stepwise processes of the structural modifications taking place in hemoglobin molecule. In conclusion, some secondary stable state of hemoglobin molecule which is highly active as an oxidative catalyst might be formed as an intermediate of hemoglobin denaturation, and once the denaturation proceeded so profoundly as to the visible degree of perturbation of hemoglobin, the oxidative activity of the hemoglobin would be lost again. It may not be surprising in these cases if the unfolded protein molecule exhibits their functional modification not by loss but by alteration of biological activity, or of catalytic activity when we speak of heme-proteins. And, as suggested by our presentation, there can be many of the criteria for the demonstration of structural modifications in protein molecule, and while one can evidence a structural change by some of these criteria, but sometimes may not by others.

It is doubtful whether salicylate affects the hemoglobin molecule as similarly as benzoate. As shown in Fig. 5, an adequate concentration of salicylate for the perturbation was lower than that of benzoate, and furthermore the optimum concentration range of salicylate was found to be narrower than that of benzoate. While, moreover, salicylate is effective to promote the choleglobin formation more strongly than the oxidative activity of hemoglobin, benzoate is effective in reverse relation. The results shown in Figs. 9 and 10 also present a question whether the effects of salicylate and benzoate are similar. Perturbation of hemoglobin by benzoate may qualitatively be of milder grade than

by salicylate.

In our previous paper, it was postulated that in the process of choleglobin formation, an intermediate compound x may be formed from hemoglobin, seemingly the protein part of the latter being mildly modified in some sort of denaturation and that this x may be playing an active role in the process of choleglobin formation. This postulation receives further verification by the present experiments.

Many investigations have been hitherto made for the elucidation of the possible relationship between the molecular constitution of hemoglobin and its function. Beside the eminent works of Pauling (*7*) and of Wyman (*8*) on the well known Bohr effect, recent investigations of Stary (*9*) and of Riggs (*10*) can be pointed out in this respect. Stary and Tekman indicated that the protein part of hemoglobin molecule might be stabilized by the attachment of CO to hemoglobin more profoundly than by that of O_2. On the other hand, Riggs has drawn attention to the fact that SH-groups in hemoglobin molecule seem to be closely connected with the mechanism of heme-heme interaction, and the fact seems to be of some importance for the elucidation of our present investigation. Also Laidler's thermodynamic studies (*11*, *12*) on the reaction mechanism of urease or pepsin seem to be suggestive to our present problem.

SUMMARY

Studies were made on the changes of catalytic activities of hemoglobin following the structural modification of the latter induced by sodium salicylate or benzoate with special reference to their influences upon the process of choleglobin formation in the hemoglobin—ascorbic acid—O_2 system. The results are summarized as follows:

1. Reaction rate of choleglobin formation, as well as of O_2 uptake, increased with increasing concentrations of salicylate or benzoate added. But, when the concentration of the perturbators increased over a definite limit, the rate decreased again.

2. The perturbation of hemoglobin molecule seemed to proceed stepwise following the increase of salicylate or benzoate concentrations. There may most probably be a secondary stable state of hemoglobin molecule.

3. The concentration of salicylate or benzoate favorable to promote the choleglobin formation was proved to be far lower than that either to bring about the spectroscopically demonstrable grade of per-

turbation of hemoglobin molecule or to promote the rate of HbO_2 autoxidation.

4. The temperature effect on the reaction of choleglobin formation under the presence of perturbators was much greater than that without perturbators.

5. From all of the results of the present experiments, it may be postulated that the above mentioned effects of benzoate and salicylate are due to the protein denaturation of a milder grade caused by them. Our previous assumption of an active intermediate compound x possibly produced in the process of choleglobin formation from hemoglobin may be accepted to be justifiable on the basis of our present experimental evidences.

The authors wish to thank Prof. K. Kaziro for his continuous guidance and encouragement during the course of the present studies.

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REFERENCES

(*1*) Kaziro, K., Kikuchi, G., Ogawa, T., and Yamada, M., *J. Biochem.*, **40**, 295 (1953)
(*2*) Tsushima, K., *J. Biochem.*, **41**, 215 (1954); **41**, 359 (1954)
(*3*) Holden, H. F., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 27 (1947)
(*4*) Heidelberger, M., *J. Biol. Chem.*, **53**, 31 (1922)
(*5*) Suzuki, M., Kajita, A., and Hanaoka, T., *J. Biochem.*, **41**, 401 (1954)
(*6*) Shukuya, R., *J. Biochem.*, **40**, 535 (1953); **41**, 469 (1954)
(*7*) Pauling, L., and Coryell, C. D., *J. Biol. Chem.*, **132**, 769 (1940)
(*8*) Wyman, J., *Advances in Protein Chem.*, **4**, 407 (1948); *J. Polymer Sci.*, **7**, 499 (1951)
(*9*) Stary, Z., and Tekman, D. S., *Bull. faculté méd Istanbul*, **14**, 18 (1951)
(*10*) Riggs, A. F., *J. Gen. Physiol.*, **36**, 1 (1952)
(*11*) Laidler, K. J., and Casey, E. J., *J. Am. Chem. Soc.*, **72**, 2159 (1950)
(*12*) Laidler, K. J., and Hoare, J. P., *J. Am. Chem. Soc.*, **72**, 2489 (1950)

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STUDIES ON THE TRICARBOXYLIC ACID CYCLE IN TUBERCLE BACILLUS

I. SOME PROPERTIES OF THE ENZYME SYSTEMS

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There is no doubt that the tricarboxylic acid (TCA) cycle is the most significant mechanism involved in the terminal respiration of animal tissues. However, the presence of TCA cycle in bacteria has been a focus of controversy (*1*). It is well known that tubercle bacillus is a typical aerobic organism. Therefore, we have supposed the occurrence of TCA cycle in tubercle bacillus and attempted to separate the enzyme system. In consequence, we have succeeded in the extraction of a number of enzymes related to TCA cycle from *Mycobacterium tuberculosis avium* in cell-free state (*2*) and demonstrated the operation of the cycle in this organism (*3*). In this paper some properties of these enzymes will be described.

EXPERIMENTAL

Methods

Preparation of Cell-Free Enzymes—Mycobacterium avium, strain T a k e o, was employed in all experiments. The medium used for growing bulk supplies has been described previously (*4*). After three days incubation at 37°, the cells were harvested and washed with distilled water. For the preparation of cell-free extract, the two different procedures were used as follows:

(A) The compact mass of bacteria was rapidly frozen, allowed to stand at −10° for 24 hours, and then thawed by incubation at 37°. This procedure was repeated again, and then the cells were ground with quarz-sand in a mortar for 30 minutes at room temperature, suspended in distilled water and allowed to stand at 5° overnight. The next day the suspension was centrifuged on 12,000 r.p.m. for 20 minutes and the supernatant fluid was used as *Enzyme Preparation A*. The similar preparation was also obtained by the grinding and autolysis of the acetone-dried cells.

(B) The fresh cells were mixed with an equal weight of distilled water and homo-

genized with Potter-Elvehjem homogenizer for about 20 minutes in ice-cold water bath. The mixture was centrifuged on 12,000 r.p.m. for 20 minutes immediately or after frozen overnight. The supernatant fluid (*Enzyme Preparation B*) was stored in frozen state for about a week.

Enzymatic Assay—The activities of various oxidases were measured by observing the oxygen uptake manometrically in a Warburg apparatus at 30°. Each substrate was added to the side arm of each flask. To the main compartment of the flask were added the enzyme preparation, 0.2 ml. of 0.5 *M* phosphate buffer at pH 6.2, other substances and distilled water to bring the total volume to 3.0 or 4.0 ml., and the experiment was carried out in air. Sometimes the procedure, in which potassium ferricyanide as election acceptor was used, was also emproyed according to the method of Stumpf *et al.* (*5*). The activity of fumarase was determined by the method of Laki and Laki (*6*). The activity of aconitase was determined by measuring the formation of citrate from added *cis*-aconitate. For the assay of oxalacetic decarboxylase activity, the rate of evolution of carbon dioxide was measured manometrically. The reaction was carried out in a Warburg flask with two side arm. The enzyme preparation, 0.2ml. of 0.5*M* phosphate buffer at pH 5.0 and distilled water were placed in the main compartment of each flask. 0.2 ml. of 0.1 *M* oxalacetate at pH 5.0 was placed in one side arm and 0.3 ml. of 9 *N* H_2SO_4 in the other. The solution of oxalacetate was prepared immediately before use. At the end of the experiment H_2SO_4 was tipped from the other side arm to stop the enzymatic reaction and to liberate the carbon dioxide retained in the fluid. A boiled enzyme blank (prepared by heating in boiling-water bath for 30 minutes) was run with all experiments. This blank value was substracted from the value observed in the presence of enzyme.

Chemical Analyses—Citrate, α-ketoglutarate, succinate, L-malate, pyruvate (α-keto acids), and oxalacetate were determined respectively according to the methods of Natelson (*7*), Krebs (*8*, *9*), Blanchard *et al.* (*10*), Silverman and Werkmann (*11*), and Cohen (*12*).

Materials—Diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), and crude coenzyme A were prepared according to the methods of Williamson and Green (*13*), LePage (*14*), and Stern *et al.* (*15*), respectively.

RESULTS

Enzyme Preparation B is able to oxidize rapidly all intermediates except α-ketoglutarate involved in TCA cycle without the addition of hydrogen-carrier or co-factor. On the other hand, *Enzyme Preparation A* oxidizes rapidly L-malate and fumarate with no addition, but citrate in the presence of Mn^{++} and methylene blue, while other intermediates such as α-ketoglutarate, succinate and pyruvate were oxidized slowly.

Therefore, for the study of the enzyme systems related to L-malate, fumarate and citrate *Preparation A* was employed, and for the other systems *Preparation B* was employed. Fig. 1 shows the oxidation of various intermediates by two preparations.

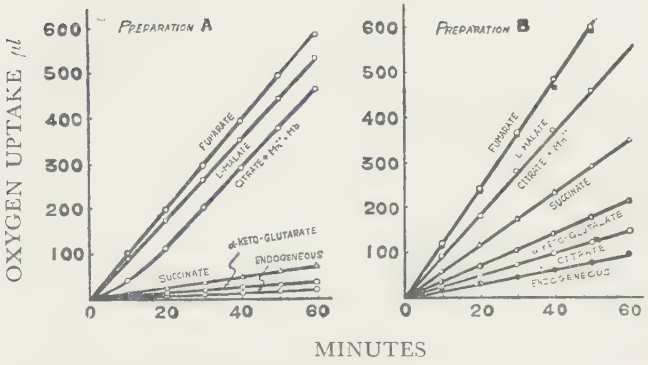


FIG. 1. The oxidation of the intermediates of TCA cycle by the cell-free extracts of *M. avium*.

Each substrate; 50 μM. 30°. In air.

(*I*) *Citric Oxidase System*

The cell-free preparation of citric oxidase is easily extracted from theacetone-dried cells or fresh cells. As shown in Fig. 2, methylene blue, Mn^{++} and DPN are required for full activity. (Fig. 2)

(*a*) *Effect of Methylene Blue*—The relation of the oxidation rate to the concentration of methylene blue is indicated in Table I. (Table I).

(*b*) *Effect of Metals*—Co^{++} and Mg^{++} have the activating action similar to Mn^{++}. Table II shows the effect of various metal ions. Ni^{++} and Fe^{++} have only slight activity, while Zn^{++}, Al^{+++} and Ca^{++} were inert. (Table II).

(*c*) *Effect of DPN*—The oxidation rate of citrate by the crude extract is not markedly increased by the addition of DPN (Table III). However, either dialysis against ice-cold distilled water for 16 hours or precipitation with 10 per cent acetic acid dissociates partially the enzyme into the apo-protein and co-factor, thus showing that DPN activates greatly the oxidation of citrate (Table III, Fig. 2b).

(*d*) *Effect of pH*—The optimum pH is the wide range of pH 5.5 to pH 8.0 (Table IV).

(*e*) *Effect of Inhibitors*—The actions of various inhibitors are given in Table V.

(*f*) *Catalyzed Reaction*—In Fig. 3, the equivalent of 1 atom of oxygen per mole of added citrate is consumed. From the data of Table VI, the reaction (1) is catalyzed by this enzyme. The formation of succinate in a small extent (Table VI) is probably due to the contamination of α-ketoglutaric oxidase in the used enzyme preparation.

(1) $\text{Citrate} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + H_2O$

The same preparation also oxidises rapidly *cis*-aconitate as well as citrate. Table VII shows the occurrence of aconitase activity in the extract. Therefore, it is considered

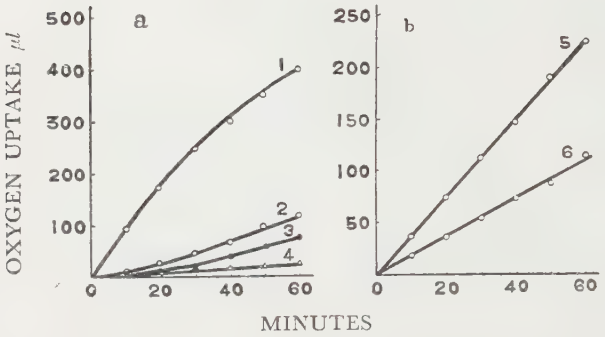


Fig. 2. The effect of methylene blue, $MnCl_2$ and DPN on the oxidation of citrate.

Fig. 2a; Warburg vessels contain 1.0 ml. of crude extract (A), 0.3 ml. of 0.2 *M* phosphate buffer (pH 6.2), 0.5 ml. of 0.1 *M* citrate, and curve 1, 0.2 ml. of 0.1 *M* $MnCl_2$ and 0.5 ml. of 0.5 per cent methylene blue. Curve 2; no $MnCl_2$, curve 3; no methylene blue, curve 4; no $MnCl_2$ and methylene blue.

Fig. 2b; Warburg vessels contain 1.0 ml. of the dialysed extract (against distilled water for 14 hours), 0.3 ml. of 0.2 *M* phosphate buffer (pH 6.2), 0.5 ml. of 0.1 *M* citrate, 0.2 ml. of 0.1 *M* $MnCl_2$ and 0.5 ml. of 0.5 per cent methylene blue. Curve 5; 0.5 ml. of DPN, curve 6; no DPN. Gas phase, air, 30°.

Table I

Effect of Concentration of Methylene Blue on Oxidation of Citrate

Concentration of methylene blue *M*	None	2×10^{-5}	2×10^{-4}	2×10^{-3}
Oxygen uptake *μl. per 90 minutes*	5	49	137	183

Warburg vessels contain 2.0 ml. of extract (A), 0.5 ml. of 0.1 *M* phosphate buffer (pH 6.0), 0.2 ml. of 0.1 *M* $MnSO_4$, 0.1 ml. of 0.5 *M* citrate, and methylene blue or water. Air, 30°.

that citrate is oxidized via *cis*-aconitate and isocitrate.

(*II*) *α-Ketoglutaric Oxidase*

This oxidase was not or little extracted by the procedure (A) including alternate freezing and thawing, drying with acetone and autolysis in a refrigerator. Also the employment of alumina and powdered glass was not efficient for grinding instead of

Table II

Effect of Various Metal Ions on the Oxidation of Citrate

Exp. No.	Mn^{++}	Mg^{++}	Co^{++}	Ni^{++}	Fe^{++}	Al^{+++}	None	Cr^{++}	Zn^{++}	Cd^{++}	Cu^{++}
I	313	254	—	180	160	145	135	—	120	80	50
II	177	170	155	48	4	—	8	7	—	—	—

Experimental conditions were the same as in Fig. 1 except metals. Each metal was used as 0.005 *M* chloride.

The values are expressed as $\mu l.$ of oxygen uptake per 60 minutes.

Table III

Effect of DPN and Boiled Enzyme on the Citric Oxidase

Enzyme preparation	Addition						
	None	DPN					Boiled enzyme
		0.1 ml.	0.3 ml.	0.5 ml.	0.8 ml.	1.5 ml.	
Original extract	155	—	—	—	203	—	—
Dialysis at 0° for 16 hours	84	115	117	141	180	190	154
Precipitate at pH 4.5 with acetic acid	196	—	—	—	—	—	384

The values are expressed as $\mu l.$ oxygen uptake per 60 minutes.

Table IV

Effect of pH on the Citric Oxidase

pH	4.8	5.5	6.2	7.0	7.5	8.0	9.8
Oxygen uptake $\mu l.$ *per 30 minutes*	80	168	167	161	148	146	70

Phosphate buffer 100 μM was employed.

quarz-sand. It appears that the procedure (B) gives the enzyme preparation with some activity. When the enzyme preparation was dialysed against distilled water for 16 hours, the oxidation of α-ketoglutarate was stimulated either by Mg^{++} or by diphosphothiamine (Table VIII). Its optimum pH was found to be between 7.0 and 8.0.

TABLE V

Effect of Inhibitors on the Citric Oxidase

Inhibitor	Final concentration	Inhibition
	M	*per cent*
Iodoacetate	6.6×10^{-3}	37
Fluoride	6.6×10^{-3}	0
Pyrophosphate	6.6×10^{-3}	0
Arsenite	1.2×10^{-3}	37
Silver nitrate	6.6×10^{-4}	45

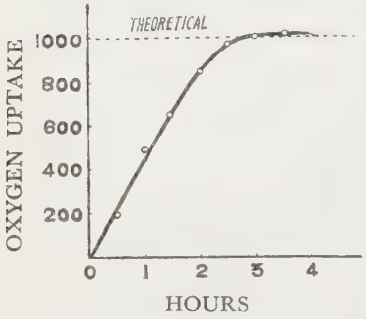


FIG. 3. Oxidation of citrate by the cell-free extract. Warburg vessel contains 2.0 ml. of crude extract (A), 0.3 ml. of 0.2 *M* phosphate buffer (pH 6.2), 0.2 ml. of 0.1 *M* $MgCl_2$, 0.5 ml. of 0.5 per cent methylene blue and 0.1 ml. of 0.4 *M* citrate.

TABLE VI

Chemical Balance of Citric Oxidation

Exp. No.	Consumption		Formation		
	Citrate	Oxygen	α-Ketoglutarate	Succinate	Carbon dioxide
	μM	μM	μM	μM	μM
I	42.8	19.5	—	—	—
II	—	9.2	—	—	19.2
III	—	36.3	57.3	8.3	—

TABLE VII

Formation of Citrate from cis-Aconitate

Reaction time *minutes*	0	5	10
Enzyme	0.00	7.51	8.19
Boiled for 3 minutes	0.00	0.00	0.00

The values are expressed as μM of citrate formed from 10 μM *cis*-aconitate. The experiments were carried out at 30° in 100 per cent N_2.

TABLE VIII

Oxidation of α-Ketoglutarate

Addition	Oxygen uptake in		
	15 minutes	30 minutes	60 minutes
None	8 $\mu l.$	12 $\mu l.$	20 $\mu l.$
$MgCl_2$, 10 μM	35	85	180
Cocarboxylase, 20 μM	21	52	107
$MgCl_2$, 10 μM+cocarboxylase, 200 γ	25	74	175

Enzyme Preparation B was dialyzed ageinst distilled water for 16 hours. α-Ketoglutarate 40 μM. Phosphate buffer 100 μM (pH 7.0). In air.

(*III*) *Succinic Oxidase*

The extraction of this enzyme is attained only by the procedure (B) similar to α-ketoglutaric oxidase. The *Enzyme Preparation B* is capable of oxidizing succinate without the addition of hydrogen carrier such as methylene blue, and remarkably inhibited by azide or malonate like the succinic dehydrogenase of animal tissues (Table IX). Recently Stone and Wilson (*16*) found that the oxidation rate of succinate by the *Azotobacter* extracts was markedly stimulated by the addition of glutamate. The similar phenomena were observed with the *Preparation B* as indicated in Table X.

TABLE IX

Effect of Inhibitors and Methylene Blue on the Succinic Oxidase

Substances	Final concentration	Oxygen uptake (*μl. per 60 minutes*)	
		None	on addition
Malonate	1.1×10^{-2} *M*	88	17
Azide	1.1×10^{-3}	103	38
Silver nitrate	3.8×10^{-4}	79	34
Methylene blue	1.25×10^{-2}	31	28

Succinate 1.7×10^{-2} *M*, phosphate buffer (pH 6.2).

The action of glutamate could not be replaced by aspartate, alanine, DPN, FAD, methylene blue or Al^{+++}. Since *Preparation B* also contained fumarase and malic oxidase, the identification of the reaction product was impossible. But the reaction catalyzed by this enzyme may proceed according to the following reaction.

TABLE X

Effect of Glutamate on the Succinic Oxidase

Exp. No.	Substrates	Oxygen uptake in 20 minutes	60 minutes	120 minutes
I	Succinate, 50 μM	8 μl.	35 μl.	74 μl.
	Glutamate, 100 μM	4	16	31
	Succinate, 50 μM+glutamate, 100 μM	34	125	262
II	Succinate, 50 μM	30	80	140
	Glutamate, 100 μM	19	55	85
	Asparate, 100 μM	5	7	10
	Succinate, 50 μM+glutamate, 100 μM	59	192	401
	,, ,, ,, ,, +aspartate, 100 μM	27	82	150

(2) $\text{Succinate} + \frac{1}{2}O_2 \longrightarrow \text{fumarate} + H_2O$

(*IV*) *Fumarase*

This enzyme was very soluble and the most easily extractable among the enzymes related to TCA cycle. The activity was indicated in Fig. 4. The optimum was at pH 7.0.

The authors attempted the further purification of fumarase contained in *Mycobacterium avium* according to the method of Laki and Laki (*6*) which has been usually taken on animal tissues and obtained some times purified enzyme preparation as the original extract.

(*V*) L-*Malic Oxidase*

Enzyme Preparation A was used. This preparation reacts directly with molecular oxygen and oxidizes L-malate. Methylene blue, cytochrome c and diphosphopyridine nucleotide have no effect on the aerobic oxidation of malate. The Michaelis-Menten constant is approximately 6×10^{-3} *M*. The rate of oxidation is essentially the same over the pH range of 6.0 to 8.0. Table XI illustrates the effect of various inhibitors. It is quite stable against distilled water at 4° for 16 hours. By the saturation of 50 per cent with ammonium sulfate the enzyme activity transfers into the part of precipitate.

On the other hand, the precipitation with 10 per cent acetic acid, which previously has been found exceedingly effective for the purification of the lactic oxidase in *M. avium* (*4*), destroys completely this enzyme. The equivalent of 1 atom of oxygen per mole of L-malate was used as shown in Fig. 5, and the equivalent of 1 mode of both carbon dioxide and α-keto acid was found per mole of substrate (Table XII).

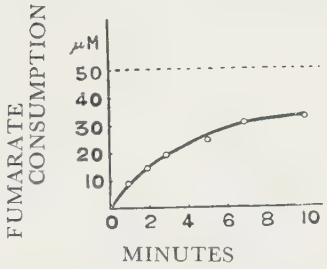


FIG. 4. Consumption of fumarate catalyzed by fumarase. Main compartment of each Warburg cup contained 0.5 ml. of the *Enzyme Preparation B* and 1.0 ml. of 0.2 *M* phosphate buffer, pH 7.0. The enzyme preparation used in this experiment was previously diluted so that no measurable oxidation of L-malate was observed for 60 minutes. One side-arm had 0.5 ml. of 0.1 *M* fumarate, and the other, 0.5 ml. of 30 per cent trichloroacetic acid. Final volume 3.5 ml.. Gas phase, 100 per cent N_2; 30°. The reaction was started by tipping fumarate from one side-arm after temperature equilibration, and after suitable time intervals the reaction was stopped by tipping trichloracetic acid from another side-arm. Then fumarate present in the cup was determined by titration with 0.01 *N* $KMnO_4$ (*6*).

TABLE XI

Effect of Inhibitors on the Malic Oxidase

Inhibitor	Final concentration	Inhibition
Silver nitrate	4.0×10^{-4} *M*	100 *per cent*
Copper sulfate	4.0×10^{-4}	59
Azide	1.6×10^{-3}	39
Iodoacetate	2.0×10^{-3}	19
Fluoride	7.4×10^{-3}	0
Malonate	7.4×10^{-2}	10
Cyanide	7.4×10^{-3}	35

Definite proof of pyruvate as the α-keto acid was made clear by the following procedure.

The collected reaction mixtures of the experiments, corresponding to these given in Fig. 5, were deproteinized with a half volume of 6 *N* HCl and centrifuged. After addition of 3 ml. of saturated 2,4-dinitrophenyl hydrazine in 2 *N* HCl into the supernatant solution, unsoluble precipitates settled out. They were collected after standing for 30 minutes and washed with 2 *N* HCl. The precipitates was taken up in ethyl acetate, extracted by addition of 10 per cent Na_2CO_3 and then reprecipi-

TABLE XII

Oxidation of L-*Malate*

L-Malate addition	10.0 μM
Oxygen consumed	4.7 μM
Carbon dioxide formed	9.0 μM
α-Keto acid formed	9.1 μM

The same experiment as in Fig. 5.

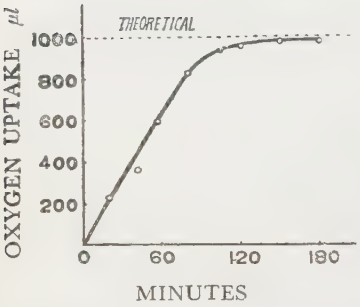


FIG. 5. Oxidation of L-malate by the cell-free extract. Warburg vessel contains 2.0 ml. extract (A), 0.3 ml. of 0.2 *M* phosphate buffer (pH 6.2), and 0.1 ml. of 0.4 *M* L-malate.

tated with HCl. This substance was identified as the 2,4-dinitrophenylhydrazone of pyruvate by m.p. (212.5–213.5°) and mixed m.p. (213.5°); the 2,4-dinitrophenylhydrazone of the synthesized pyruvic acid melted at 213.0°. Summarizing the preceeding qualitative and quantitative results, it is shown that the oxidation of L-malate by this enzyme preparation proceeds according to the following reaction.

(3) L-Malate$+\frac{1}{2}O_2 \longrightarrow$ pyruvate$+CO_2+H_2O$

The oxidase is also able to use ferricyanide, methylene blue, and other dayes as electron acceptor (Table XIII). The most rapid rate of oxidation is attained with ferricyanide as an electron acceptor.

TABLE XIII

Oxidation of Malate by Various Oxidants

Electron acceptors	Exp. No. 1	Exp. No. 2*
Oxygen	251 $\mu l.$ O_2	53 $\mu l.$ O_2 *per hr.*
Methylene blue, 10 μM	96 $\mu l.$ CO_2	—
Ferricyanide, 100 μM	—	504 $\mu l.$ CO_2

* The enzyme preparation was extracted from the acetone.dried cells.

(*VI*) *Oxalacetic Decarboxylase*

The above mentioned *Preparation A* catalyzes the rapid decarboxylation of oxalacetate. The pH optimum is about pH 5.0, while the non-enzymatic decarboxylation of oxalacetate increased in a linear fashion from acid to alkali range. The effect of metals was examind. Undialyzed enzyme was not activated by any metal, but when the extract was dialysed against distilled water or pyrophosphate for 15 hours above, it was observed that Mn^{++} and Mg^{++} had activating effects. Table XIV shows that Co^{++} is inactive, although it increases exceedingly the decarboxylation in the presence of the boiled enzyme. On the other hand, Plaut and Lardy (*17*) found that the oxalacetic decarboxylase of *Alzotobacter vinelandii* was activated by Mn^{++}, CO^{++} and Zn^{++}, and slightly by Mg^{++}. Data in Table XV indicate that the oxalacetic decarboxylase of *M. avium* is different from the enzymes of other sources. Malate, malonate and ATP have little or no inhibition effect. Ochoa and Weisz-Tabori (*18*) found that malate inhibits the decarboxylation of oxalacetate by the purified preparation of "malic enzyme" from pigeon liver. The inhibitions of malonate and ATP were respectively found by Evans *et al.* (*19*), and Plaut and Lardy (*17*).

TABLE XIV

Effect of Metals on Decarboxylation of Oxalacetate

Metal	Carbon dioxide evolved in minutes		
	With enzyme (a)	With boiled enzyme (b)	(a)—(b)
None	52 *µl.*	25 *µl.*	28 *µl.*
Mg^{++}	168	101	67
Mn^{++}	133	80	53
Ni^{++}	77	58	19
CO^{++}	202	228	0
Cr^{++}	56	31	25
Fe^{++}	49	21	18

The enzyme was dialyzed against ice-cold distilled water for 16 hours. Each metal was used as chloride in the concentration of 0.005*M*.

(*VII*) *Citric Acid Synthesis System*

Recently the condensation mechanism of citrate has been clarified by Ochoa (*15*, *20*) and other investigators. The wide distribution of the "condensing enzyme" in microorganisms including *M. tuberculosis v. hominis* has been shown in their report (*20*). In the previous communication (3), we have found the formation of citrate from C_4-dicarboxylic acids by the acetone-dried cells of *M. avium*. In this paper,

Table XV

Effect of Various Inhibitors on Oxalacetic Decarboxylase

Inhibitors	Final concentration	Inhibition
Silver nitrate	4.8×10^{-4} *M*	100 *per cent*
Pyrophosphate	5×10^{-3}	40
ATP	5×10^{-3}	0
Iodoacetate	1×10^{-2}	0
Malonate	2.5×10^{-2}	0
Cyanide	2.5×10^{-2}	20
Malate	2.5×10^{-2}	18

the formation of citrate using the cell-free preparation is indicated. *Preparation B* contains the active enzyme systems involved in the citrate synthesis, while *Preparation A* is quite inert. Table XVI shows oxygen molecule is required for the system. Either malate or fumarate can replace oxalacetate as in Tables XVI and XVII.

Table XVI

The Formation of Citrate by Cell-free Enzyme Preparation

Exp. No.	Gas phase	Substrate	Citrate formed
I	100% N_2	Oxalacetate, 80 μM	1.33 μM
	Air	// , // //	24.12
II	//	None	0.00
	//	Acetate, 50 μM	0.00
	//	Pyruvate, // //	1.07
	//	L-Malate, 25 μM	3.84
	//	L-Malate, 25 μM+acetate, 50 μM	4.43
	//	// , // // +pyruvate, 50 μM	4.16
	//	// , // // + // , // // + acetate, 50 μM (with boiled enzyme)	0.00

Each Warburg vessel contains 2.0 ml. of *Enzyme Preparation B*, 0.2 ml. of 0.5 *M* phosphate buffer (pH 6.2), substrates and water to bring the total volume to 3.0 ml. After incubation at 30° for 60 minutes, 1.0 ml. of 10 per cent trichloroacetic acid was added from a side-arm, the mixture was centrifuged and citrate was determined.

The formation of citrate is found apparently from C_4-dicarboxylic acids and the addi-

TABLE XVII

Effect of Crude CoA and Malonate on the Citrate Formation

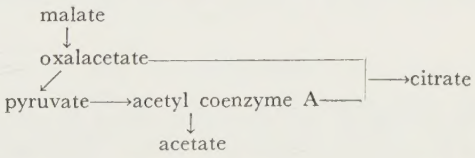
Exp. No.	Addition	Citrate formed
I*	Oxalacetate, 60 μM	3.20 μM
	,, , +CoA	4.69
II*	Fumarate, 100 μM	0.78
	,, , ,, +CoA	2.23
	,, , ,, +CoA (with boiled enzyme)	0.00
III	Oxalacetate, 40 μM	8.07
	Malonate, 200 μM	0.00
	Oxalacetate, 40 μM+malonate, 200 μM	16.87
IV	Fumarate, 40 μM	2.99
	,, , ,, +malonate, 200 μM	6.25

Conditions as in Table XVI.

* The enzyme was dialyzed against distilled water for 18 hours.

tion of acetate causes an only slight increase. Added pyruvate has no effect. This is probably due to that *Preparation B* involves the active oxalacetic decarboxylase and pyruvic oxidase, and a sufficient amount of active C_2-compound is given for the partner of oxalacetate. With the dialyzed enzyme the activation-effect of the crude coenzyme A is observed (Table XVII). The remarkable stimulation of citrate formation by malonate seemed probably to be due to that malonate prevents the citrate decomposition. In *M. avium*, the formation of citrate is suggested to occur by the Ochoa's mechanism as formulated as follows:

(4)



The addition of streptomycin (700 μ per ml.) has no effect upon the formation of citrate.

DISCUSSION

From the above experimental results, it was shown that *M. avium* possessed all the enzyme systems related to TCA cycle and these enzyme activities were comparatively high. These facts suggest strongly that these enzymes construct TCA cycle, which operates in the terminal

respiration of this bacterium.

In recent years, a number of investigations on the terminal oxidation of bacteria have been undertaken (*1*), but the nature of the mechanism has been still obscure. Karlsson and Barker (*21*) showed the evidence to support that *Azotobacter agilis* oxidizes acetate without the participation of TCA cycle, by the method of simultaneous adaptation, or isotopic technique with mutant strain isolated from an X-ray treated culture. Barron *et al.* (*22*) came to the conclusion that *Corynebacterium creationovorans* oxidizes acetate *via* the dicarboxylic acid cycle. The difficulties of the study on the bacterial terminal oxidation are partly responsible to the fact that cell-free extract of bacteria generally do not exhibit appreciable aerobic activity. In this respect our enzyme preparation will furnish evidences strongly supporting the occurrence of TCA cycle in bacteria. When the above experiments were in progress, a review by Edson (*23*) appeared reporting the extraction of the enzymes involved in TCA cycle from *Mycobacteria*. Their findings agree well with our results (*23*, *24*).

Further, Stone and Wilson (*16*) obtained the cell-free extracts of *Azotobacter vinelandii* having the ability to oxidize acetate, pyruvate, and the acids of TCA cycle. We have also investigated the possibilities of the presence of other pathway in the terminal respiration of *M. avium*, and found that succinate was remarkably formed from malate (or fumarate) either aerobically or anaerobically by *Preparation B*. The evidence suggested strongly that the mechanism of succinate formation proceeded not *via* an oxidative TCA cycle (*25*). The nature of this mechanism will be described in the next communication.

SUMMARY

We have separated from *Mycobacterium avium* the enzymes involved in the TCA cycle, namely aconitase, citric (probably isocitric), α-ketoglutaric, and succinic oxidases, fumarase, L-malic oxidase, oxalacetic decarboxylase and citrate synthesis enzymes in cell-free state, and the extraction procedures and some properties of these enzymes were described here.

It is concluded that the TCA cycle operates actively in the terminal respiration of *Mycobacterium avium*.

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REFERENCES

(*1*) Ajil, D.J., *Bacteriol. Rev.*, **15**, 211 (1951)
(*2*) Kusunose, M., and Kusunose, E., The 4th Meeting of the Kinki Association for Tuberculosis, (1951)
(*3*) Kusunose, M., Kusunose, E., and Yamamura, Y., *J. Biochem.*, **39**, Proceedings of the 24th General Meeting of Japanese Biochemical Society, xxxiv (1952); Kusunose, M., and Kusunose, E., *Kekkaku*, **28**, 34 (1953)
(*4*) Yamamura, Y., Kusunose, M., and Kusunose, E., *J. Biochem*, **39**, 227 (1952)
(*5*) Stumpf, P.K., Zarudnaya, K., and Green, D.E., *J. Biol. Chem.*, **167**, 817 (1947)
(*6*) Laki, E., and Laki, K., *Enzymologia* **9**, 139 (1941)
(*7*) Natelson, S., Pincus, J.B., and Lugovoy, J.K., *J. Biol. Chem.*, **175**, 745 (1948)
(*8*) Krebs, H.A., *Biochem. J.*, **32**, 108 (1938)
(*9*) Krebs, H.A., *Biochem. J.*, **31**, 2095 (1937)
(*10*) Blanchard, M.L., Korkes, S., Campillo, A. del., and Ochoa, S., *J. Biol. Chem.*, **187**, 875 (1950)
(*11*) Silverman, M., and Werkmann, C.H., *J. Biol. Chem.*, **138**, 35 (1941)
(*12*) Cohen, P.P., Manometric techniques and tissue metobolism, edited by Umbreit, W.W., Burris, R.H., and Stauffer, J.F., p. 175 (1949)
(*13*) Williamson, S., and Green, E.D., *J. Biol. Chem.*, **135**, 345 (1940)
(*14*) LePage, G.A., Manometric techniques and tissue metabolism, edited by Umbreit, W.W., Burris, R.H., and Stauffer, J.F., p. 204 (1949)
(*15*) Stern, J.R., Shapiro, B., Stadtman, E.R., and Ochoa, S., *J. Biol. Chem.*, **193**, 703 (1951)
(*16*) Stone, R.W., and Wilson, P.W., *J. Bacteriol.*, **63**, 619 (1952)
(*17*) Plaut, G.W.E., and Lardy, H.A., *J. Biol. Chem.*, **180**, 13 (1949)
(*18*) Ochoa, S., and Weisz-Tabori, E., *J. Biol. Chem.*, **174**, 123 (1948)
(*19*) Evans, E.A., Jr., Venvesland, B., and Slotin, L., *J. Biol. Chem.* **147**, 771 (1943)

(*20*) Ochoa, S., Stern, J.R., and Schneider, M.C., *J. Biol. Chem.*, **193**, 691 (1951); Stern, J.R., and Ochoa, S., *J. Biol., Chem.*, **179**, 491 (1949)
(*21*) Karlsson, J.L., and Barker, H.A., *J. Biol. Chem.*, **175**, 913 (1948)
(*22*) Barron, E.S.G., Ardao, M.I., and Hearon, M., *Arch. Biochem.*, **29**, 130 (1950)
(*23*) Edson, N.L., *Bacteriol. Rev.*, **15**, 147 (1951)
(*24*) Blakley, R.L., *J. Bacteriol.*, **64**, 609 (1952)
(*25*) Kusunose, M., Nagai, S., Kusunose, E., and Yamamura, Y., The 25th General Meeting of the Japanese Biochemical Society (1953)